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Identification and in Vitro Evaluation of New Antileishmanial Drugs Using Bioinformatics

By

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Dedication

To my parents …

To my husband …

To my sons …

To my brothers …

To my sisters …

To whom I love …

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Abbreviations

ACP:	Acyl carrier protein
AD:	Alternate day
Amb:	amBisome
BLAST:	Basic local alignment search tool
bp:	Base pair
C:	complement
CL:	Cutaneous leishmaniasis
CM:	Complete media
CR:	Complement receptor
DAT:	Direct agglutination test
DCL:	Diffuse Cutaneous leishmaniasis
DHFR:	Dihydrofolate rteductase
DMEM:	Dulbecco's modified eagle's medium
DMSO:	Dimethyl sulfoxide

DNA:	Deoxy ribonucleic acid
ELISA:	Enzyme linked immunosorbent assay
Gp63:	Glycoprotein63
IFAT:	Immunoflourescent antibody test
IFN?:	Gamma interferon
IL:	interlukin
IM:	Intramuscular
INH:	Isoniazid
IV:	intravenous
KDNA:	Kinetoplastida DNA
MCL:	Mucocutanous leishmaniasis
ML:	Mucosal leishmaniasis
NNN:	Novy-Macneal-Nicolle medium
NO:	Nitric oxide
PCR:	Polymerase chain reaction

PKDL:	Post Kalazar dermal leishmaniasis
PTR1:	Pteridine reductase
Rpm:	Revolution per minute
SSG:	Sodium stibogluconate
TBE:	Tris boric FDTA
TH:	T helper cell
THP:	Human acute monocytic leukemia cell
TS:	Thymidylate synthase
VL:	Visceral leishmaniasis
WHO:	World heasslth organization

ABSTRACT

Introduction: *Leishmaniasis* is a major public health problem in several parts of the world. It is caused by the obligate intracellular protozoan parasite of the genus *leishmania* and is transmitted by the female sand fly of the genera phlebotomus and lutzomyia. It presents in different clinical forms: cutaneous (CL) diffuse cutaneous (DCL), Visceral (VL), mucocutaneous (MCL), mucosal (ML) or post kalazar dermal leishmaniasis (PKDL). Leishmaniasis is difficult to treat and there is increasing resistance developing against the currently available drugs which are toxic, expensive, given by injection and requires hospitalization for long duration.

Objectives: The aim of this study was to identify and evaluate selected drugs used for treatment of other diseases for antileishmanial activity using comparative bioinformatics and in vitro human macrophage (THP1) infection assay.

Methods: Basic Local Alignment Search Tool (BLAST) was used to determine protein homology of the target sequence of isoniazid in *Mycobacterium tuberculosis* and *Leishmania* parasites. Similar search was done to determine protein homology of target sequence of sulfadoxine pyrimehamine in *Plasmodium* and *Leishmania* parasites.

Based on the determined protein homology, antileishmanial activity of isoniazid, isoniazid plus sodium stibogluconate (SSG), isoniazid plus liposomal amphotericin B (AmBisome), sulfadoxine pyrimethamine combination was

evaluated using in vitro human macrophage (THP1) infection assay. The data was statistically analyzed using paired sample t test.

Results: Alignment of isoniazid target sequence in mycobacterium tuberculosis and leishmania major and donovani showed 50% maximum identities while alignment of Sulfadoxine pyrimethamine target sequence in plasmodium falciparum and Leishmania donovani and Leishmania major showed 53% maximum identities.

Significant antileishmanial activity of isoniazid against both *Leishmania major* and *Leishmania donovani* at 0.1, 0.2 and 0.4µg/ml concentrations was detected by decreasing the number of amastigotes compared to the negative control. Significant synergistic effect when isonaizid was used in combination with sodium stibogluconate and with liposomal amphotericin B was detected by increasing the percentage of killing compared with that of each drug alone.

Sulfadoxine pyrimethamine combination showed minimum antileishmanial activity at 1.9 pyrimethamine and 38.1 sulfadoxine, 2.9 pyrimethamine and 57.1 sulfadoxine, 3.8 pyrimethamine and 76.2 sulfadoxine concentrations compared with SSG.

Conclusion: Isoniazid showed significant antileishmanial activity and significantly enhanced the antileishmanial effect of SSG and amBisome, while Sulfadoxine pyrimethamine combination had minimum antileishmanial activity.

ملخص الاطروحة

مقدمة: يعتبر مرض اللشمانيا من مشاكل الصحة العامة الرئيسية في اجزاء مختلفة من العالم ويسببه طفيل أولي يعيش داخل الخلايا إجبارياً من جنس الليشمانيا. ينتقل المرض بواسطة أنثى الذبابة الرملية من جنس فلوبوتوماس ولوتزوميه. يظهر المرض باشكال سريرية مختلفة: جلدي، جلدي منتشر، حشوي، مخاطي جلدي، مخاطي والجلدي التالي للحشوي. من الصعب معالجة مرض الليشمانيا كما أن هناك زيادة في تطور مقاومة الطفيل للأدوية الموجودة حالياً والتي تتصف بانها سامة، باهظة الثمن، تعطى عن طريق الحقن وتحتاج الي تنويم المريض في المستشفى لفترة طويلة.

الاهدف: الهدف من هذه الدراسة التعرف وتقييم بعض الأدوية المستخدمة لعلاج بعض الأمراض الأخرى كعلاج لمرض اللشمانيا وذلك باستخدام المعلوماتية الحيوية المقارنة و تقنية عدوى البلاعم معملياً.

طريقة الدراسة: تم استخدام تقنية المحاذاة المحلية الاساسية (BLAST) للتعرف علي مدي تماثل البروتين في السلسلة المستهدفة لعقار ايزونيازد في المتفطرة السلية واللشمانيا الجلدية و الدونوفانية بالاضافة الي التعرف علي مدى تماثل البروتين في السلسلة المستهدفة لعقار سلفادوكسين وبايرمثامين في طفيل البلازموديوم واللشمانيا. تم تحديد الفعالية المضادة لللشمانيا لعقار ايزونيازد لوحده وعقار ايزونيازد مع صوديوم ستبوجلوكونيت وعقار ايزونيازد مع الامبسوم وللعقار المكون من سلفادوكسين وبايرمثامين معا بواسطة تقنية عدوى البلاعم معملياً. كما تم تحليل النتائج إحصائيا بواسطةt test.

نتيجة الدراسة: اثبتت الدراسة ان هنالك تماثل في السلسلة المستهدفة لعقار ايزونيازد في المتفطرة السلية واللشمانيا الجلدية و الدونوفانية بنسبة 50% كحد أعلى. وتماثل في السلسلة المستهدفة لعقار سلفادوكسين وبايرمثامين في طفيل البلازموديوم فالسبرم واللشمانيا الجلدية واللشمانيا الدونوفانية بنسبة 53% كحد أعلى.

اثبت عقار ايزونيازد فعاليته ضد اللشمانيا الجلدية واللشمانيا الدونوفانية في تركيز 0.1, 0.2 و 0.4 مايكروغرام / مليليتر من خلال خفض عدد طفيل اللشمانيا مقارنة مع المراقبة السلبية. وهذا العقار يزيد من فعالية صوديوم ستبوجلوكونيت و عقار لايبوسومال امفوتيرسين ب ضد اللشمانيا من خلال زيادة النسبة المئوية للقتل مقارنة مع كل عقار لوحده. أما العقار المكون من سلفادوكسين وبايرمثامين فقد أظهر فعالية أقل في تركيز 1.9 بايرمثامين و 38.1 سلفادوكسين, 2.9 بايرمثامين و57.1 سلفادوكسين و3.8 بايرمثامين و76.2 سلفادوكسين مايكروغرام / مليليتر مقارنة مع عقار صوديوم ستبوجلوكونيت.

خلاصة الدراسة: عقار ايزونيازد له فعالية ضد اللشمانيا وله تاثير تآزري اذا تم استخدامه مع صوديوم ستبوجلوكونيت او الامبسوم. أما العقار المكون من سلفادوكسين والبايرمثامين فله فعالية اقل ضد اللشمانيا.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION AND LITERATURE REVIEW

Leishmaniasis is a major public health problem in several parts of the world. It is a group of diseases endemic in 88 countries in Asia, Africa, Europe and Latin America. The annual incidence of cutaneous leishmaniasis is estimated at 1.5 -2 millions and 500.000 new cases of visceral leishmaniasis are estimated (WHO 2004). Both the geographic distribution and case numbers are reported to be on increase (WHO 2000).

Leishmaniasis is caused by the obligate intracellular protozoan parasite of the genus *leishmania* and is transmitted by the female sand fly of the genera phlebotomus and lutzomyia (Elnaiem *et al.*, 1998; Molyneux and Ashford, 1983). It presents by different clinical forms: cutaneous (CL), diffuse cutaneous (DCL), Visceral (VL), mucocutaneous (MCL), or mucosal (ML) and post kalazar dermal leishmaniasis (PKDL). In CL and MCL the parasites generally remain restricted to the skin or skin and mucosa respectively. CL lesions may persist for long periods, but tend to heal spontaneously, whereas a greater tendency to destructive changes is seen in MCL infections. In contrast, in VL the leishmania organisms can invade the entire mononuclear phagocytic system in various organs (spleen, liver, lymph nodes, bone marrow, blood monocytes, etc.), causing infections that are normally lethal without treatment. The course of an infection is apparently dependent on the activation of various T lymphocyte subpopulations by Leishmania antigens. Activation of TH1 cells involves production of IFN?, which

activates macrophages that exert a protective effect by killing Leishmania organisms by nitric oxide (NO) mediated mechanism. On the other hand, when TH2 cells are activated large amounts of IL-4 and IL-10 are produced, which inhibit NO activity, thus reducing or even preventing elimination of the parasites. In CL, high concentrations of IFN? were produced while in severe cases of VL the levels of IL-4 and IL-10 were high and IFN? concentration was low. The situation is similar in severe forms of MCL.

Diagnosis of leishmaniasis is based on direct demonstration of leishmania parasites on smears made from aspirate from cutaneous lesions or lymphoid organs. (Weiss, 1995; Osman *et al.*,1997), several immunological methods such as enzyme linked immunesorbent assay (ELISA), the direct immunoflourescent antibody test (IFAT) (Zuckerman, 1975) and direct agglutination test (DAT) (Harith *et* al., 1986), were developed and used for diagnosis of leishmaniasis. Polymerase chain reaction (PCR) amplification of parasite DNA is widely used technique for diagnosis and characterization of leishmania species (Chance *et al*., 1974). Leishmaniasis is difficult to treat and there is increasing resistance developing against the currently available drugs (Davidson, 1999). Sodium stibogluconate (SSG) is the first line of treatment of visceral leishmaniasis in Sudan. Liposomal amphotericin B is the second line treatment. Other drugs such as meltifosine and paromomycin are under evaluation.

1.1.1 Clinical forms of leishmnaiasis

Specific clinical manifestations are generally associated with particular *leishmania* species, but different clinical forms can be caused by the same species.

1.1.1.1 Cutaneous leishmaniasis (CL)

Cutaneous leishmaniasis (CL) in Sudan is caused by *Leishmania major* and *Leishmania donovani* (Elamin *et al.,* 2008). The disease is endemic in many parts of the country. Clinically CL patients usually present with papules, nodules or noduloulcerative lesions mainly on the exposed parts of the skin, particularly the limbs. The face is less frequently affected. Lesions on the face are more frequently encountered in children than in adults. Other sites affected include the trunk and less commonly the scalp, buttocks, eyelids and genitalia. (Elhassan and Zijlstra, 2001).This is the simplest form of the disease and can heal spontaneously but can persist for many years.

1.1.1.2 Diffuse cutaneous leishmaniasis (DCL)

It is a disfiguring but a rare form of cutaneous disease. It is characterized by multiple non-ulcerative nodules or plaques. This form of the disease does not self heal and is very difficult to treat (Bryceson, 1996).

1.1.1.3 Visceral leishmaniasis (VL)

Visceral leishmaniasis, also known as (Kala-Azar), has been among the most important health problems in Sudan. The main endemic focus extends from the Upper Nile state, north to the White Nile state, east to the Blue Nile and north east to Gadarif State. The global incidence of VL is estimated to be 0.5 millions, 90% of VL cases occur in Bangladesh, Brazil, India and Sudan (Desjeux, 2004). 95% of VL patients die resulting in at least 500,000 deaths per year worldwide. It is the most severe form of leishmaniasis and it has a mortality rate of almost 100% within one year (Desjeux 2004).

Visceral leishmaniasis in Sudan is caused by *leishmania donovani*; the disease is characterized by chronic fever, weakness, weight loss, hepatosplenomegaly, lymphadenopathy and anemia.

1.1.1.4 Post kala-azar dermal leishmaniasis (PKDL)

Visceral leishmaniasis may develop after the treatment into dermatosis commonly known as post kala-azar dermal leishmaniasis (PKDL) which is characterized by skin rashes and lesions. In Sudan 56%-62% of VL patients who have been successfully treated for VL develop PKDL (Elhassan and Zijlstra, 2001). In about 15% of PKDL cases the disfiguring lesions persist sometimes for many years. Although the lesions heal spontaneously after few weeks, the lesions can lead to

severe facial scarring leading to social stigma confused with leprosy (Musa *et al.*, 2005).

1.1.1.5 Mucocutaneous leishmaniasis (MCL)

The term mucosal leishmaniasis is used in Sudan rather than mucocutaneous leishmaniasis. Sudanese mucosal leishmaniasis is chronic infection of upper respiratory tract and /or oral mucosa caused mainly by *L.donovani* and less frequentely by *L.major* (Ghalib *et al.,* 1992). The disease occurs in areas of the related country endemic for VL particulary among Masalit and other closely tribes in western Sudan (Elhassan and Zijlstra, 2001).

The symptoms depend on the location of the lesion. Patients may present with pain, but this is usually mild. Those with nasal leishmaniasis complain of nasal obstruction, discharge, bleeding and deformity of the nose. Patient with oral and pharyngeal disease have a sensation of fullness in the mouth and may have difficulty in mastication and swallowing. Bleeding may occur particularly when the gums are affected. Toothache may be the first symptom. The teeth may become loose and are sometimes shed spontaneously (Abbas *et al.*, 1992). Hoarseness of voice, bleeding and cough occur when the larynx is affected.

Fig (1): Distribution of leishmaniasis in Sudan and South Sudan adopted from edoc.hu.berlin.de. (# Endemic areas of visceral, \\\ endemic areas of cutaneous leishmaniasis).

1.1.2 The life cycle of leishmania parasite

In human and other vertebrates, leishmania resides in phagocytic cells (macrophages, monocytes and langerhans cells) as amastigote form which is round to oval in shape, 2-5 µm in diameter with a nucleus and rod shaped kinetoplast. The amastigote stages of the parasite is ingested by the sand flies (*Phlebotomus oriental* and *Phlebotomus papatasi*) (Ashford *et al.*, 1992; Elnaiem *et a.,l* 1998) with blood meal, then transforms in the fly gut into slender flagellate promastigote forms 10-15µm long which multiply and migrate back into proboscis.

When infected sand flies take another blood meal, the promastigote forms are inoculated into new hosts. In the mammalian host the promastigotes invade macrophages, and then they transform into amastigote form and reproduce by binary fission. The amastigotes are then released from macrophages by exocytosis (Molyneux and Killick-Kendrick, 1987).

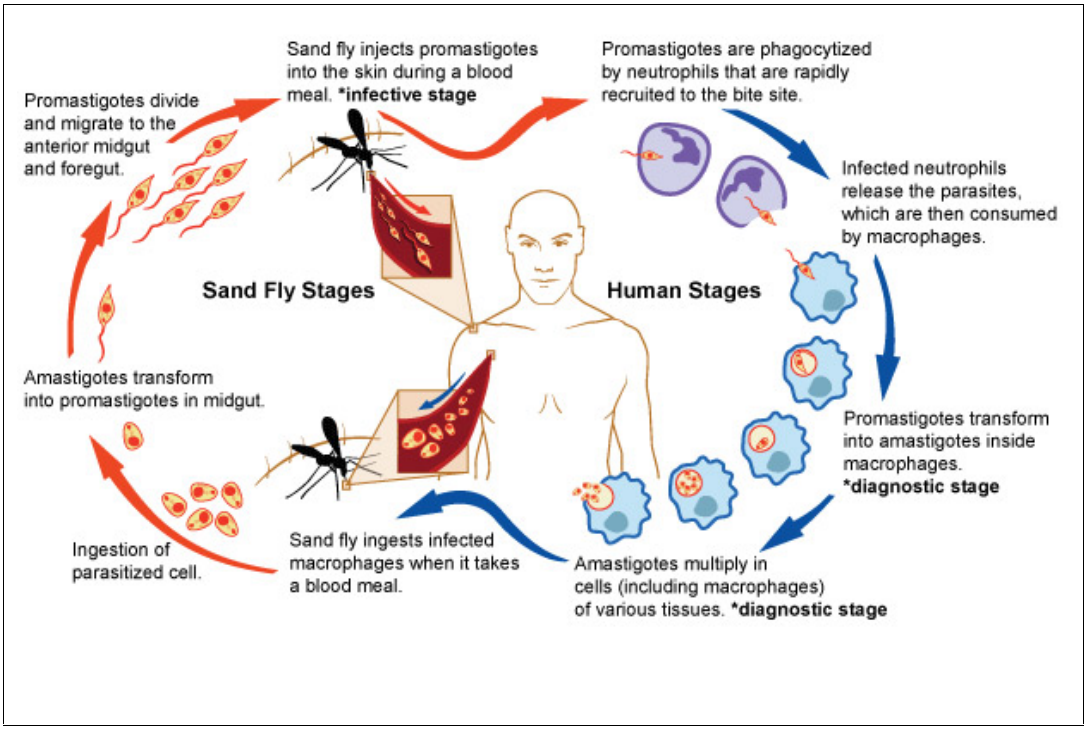

Fig (2): life cycle of leishmania parasites adopted from niaid.nih.gov

1.1.3 leishmania parasite and macrophage interaction

Leishmania parasite has a number of surface molecules that play important roles in host parasite interaction such as the attachment and the uptake of the parasite by the macrophage. These are the acid phosphate and the lipophosphoglycans (LPG) such as glycoprotein GP63. The LPG is restricted to the promastigotes. It protects the promastigotes from host complement lysis and it has role in the establishment of the parasite in the macrophage (Mc Conville and Blackwell, 1991). When the infective promastigotes injected into the host dermal tissue, they generate chemotactic activity for monocytes via GP63 by activating the complement with production of C5a and C3b (Sorensen *et al*., 1989; Bryceson, 1996). C3b is deposited on the parasite surfaces and is recognized by a macrophage receptor (Wilson and Pearson, 1988). The promastigote's GP63 is involved in the binding of promastigotes to macrophage through a receptor-mediated mechanism. The macrophage receptors that are involved are; the complement receptors (CR1) and (CR3) (Talamas-Rohana *et al*., 1990). CR3 binds directly to GP63, which has been shown to be the receptor for the promastigote surface glycoprotein, GP63 (Russel and Wright, 1988). In addition, mannose-fucose and lectin-like receptors play major role in the attachment of the parasite to the macrophage surface (Blackwell *et al*., 1985). Then the promastigotes will be taken up by macrophages and transform into amastigotes which are internalized in the phagosome and then the lysosome of the

macrophage fuses with the phagosome to form a phagolysosome containing the parasite.

1.1.4 Treatment of leishmaniasis

Sodium stibogluconate (SSG) is the classic treatment of all forms of leishmaniasis (Berman 2003). Liposomal amphotericin B is very potent agent for visceral leishmaniasis. Additional antileishmanials include paromomyin, miltefosine and others.

1.1.4.1 Sodium stibogluconate (SSG)

SSG is a pentavalent antimonial and it is the classic treatment for all forms of leishmaniasis, including the cutaneous disease. To be absorbed, antimonials must be delivered parentrally, either intravenously or intramuscularly. The standard dose for New World cutaneous disease is 20 mg antimony/kg/day for 20 days. The treatment needed for cutaneous disease is non parenteral therapy, or a shorter course of parenteral therapy both of which are more appropriate than 20 days of antimony injections for this ultimately self healing skin ulcer. The standard dose for visceral disease is 20 mg antimony /kg/day for 28 days (Berman 2003).

Antimonials are known to inhibit glycolytic enzymes and fatty acids oxidation in amastigote form. Severe side effects were reported such as pancreatitis which is the cause of nausea and abdominal pain experienced by many patients. Other side effects include pancytopenia, peripheral neuropathy and cardio toxicity (Brummit,

1996). These compounds continued to be used successfully to treat millions of patients throughout the world for almost half century, but reports of unresponsiveness to the standard 10 mg /kg body weight of SSG started in 1970s. However, the disease remained treatable with higher doses of SSG 20mg/kg body weight but many cases were reported to have developed resistance (Thakur *et al.*, 1991).

1.1.4.2 **Amphotericin B**

It is antifungal agent discovered in 1956 from a bacterium of genus streptomyces. Amphotericin B binds to cell wall sterols but preferentially to ergosterol which is the major cell memberane sterol of fungi as well as leishmania. Amphotericin B has been given to large number of patients with kala-azar who were clinically resistant to SSG and pentamidine. In India 99% of patients were cured with standard regimen of 20 injections (1mg/kg alternate day (AD)) of amphotericin B (Mishra, 1992). Amphotericin B has infusion related side effects such as fever, chills, bone pain, cardiac arrest and delayed side effects such as hypokalaemia and nephrotoxicity (Berman, 1997; Sundar, 2001). These undesirable effects are reduced by association of Amphotericin B with the liposomes (liposomal Amphotericin B; Ambisome ;) (Singh and Sivakumar, 2004). This association not only reduces the drug toxicity but also increases their efficiency and concentration in tissues. Liposomal amphotericin B is a very potent agent for visceral leishmaniasis and is the drug of choice for antimony resistant disease. But

unfortunately the production is very expensive which make their use difficult in poor country (Golenser *et al*., 1999).

1.1.4.3 **Paromomycin**

Paromomycin (aminosidine) is aminoglycoside used for the treatment of bacterial diseases. However, it has been found to have broad antiparasitic activity not shared with other aminoglycosides. Injecetable paromomycin is used for visceral leishmaniasis at dosage of 14-16 mg/kg/day given for up to 3 weeks. Paromomycin at a dose of 16 mg/kg/day for 21 days cured 24 out of 27 (89%) Indian patients with visceral disease, in a region where there was antimony resistance such that a concomitant antimony treated group had a 69% cure rate (Thakur *et al*., 2000). But hence as an amino glycoside, it has the potential risk for renal toxicity and eighth cranial nerve toxicity.

1.1.4.4 **Miltefosine**

Miltefosine is alkyl phosoholipid which was developed as anti tumor agent. It stimulates the hematopoietic and immune system, with T cells and macrophage activation and with the increase of the interferon-? production, thus helping against Leishmania infection (Escobar *et al*., 2001), therefore has recently been approved for the treatment of visceral leishmaniasis in adult at dose of 100 mg (2.5 mg /kg) for 4 weeks. It is also being used in the treatment of cutaneous leishmaniasis. Preclinical studies using the same dosage as for anticancer

treatment indicated that the drug had teratogenic effects, renal toxicity and adverse effects on fertility in males. The dosage of the drug required for the treatment of VL is however far less than that required for management of cancer and consequently has reduced adverse reactions. The result of phase III clinical trials of miltefosine in India have shown that this oral drug is very effective for treating VL in both adults and children and has limited side reaction (Berman, 2003).

1.1.4.5 **Pentamidine**

This drug was primarily used for treating *Pneumocystis carinii* pneumonia. The exact mechanism of action of pentamidine isethionate is not known, but some workers hypothesized that this polyamine compound acts on the kinetoplast DNA and inhibits its functions (Singh and Sivakumar, 2004). The pentamidine treatment was successfully used in the late 1970s and early 1980s and a cure rate of 98.8% was reported without any relapse (Jha, 1983). The regimen consisted of 4mg/kg given three times a week for 3–4 weeks (10–12injections). However, the success rate started declining in the 1980s when even after 20 injections only 75.2% of patients were cured (Thakur *et al.*, 1991). The repeated administration of 2 mg pentamidine isethionate/kg every other day for 7 days was studied for the treatment of cutaneous disease in Colombia. This regimen was 96% effective. To decrease the dosing further, 2 mg/kg pentamidine was administered every other day for only 4 days, but this decreased the cure rate to 84%. However, a slightly

higher dose (3 mg/kg/IM) administered on 4 alternate days resulted in a 96% cure rate for 51 evaluable patients (Soto *et al.,* 1994). The low-dose, short-course regimens for cutaneous leishmaniasis commonly result in myalgias, pain at the injection site, nausea, headache and less commonly result in a metallic taste, a burning sensation, numbness, and hypotension. Reversible hypoglycemia occurred in about 2% of cases. The incidence and severity of these side effects are higher when the high dose, long course regimens are used for treatment of visceral disease.

Because of the high rate of toxicity of pentamidine and recent reports of emergence of drug resistance, this drug is rarely being used for visceral disease. For cutaneous disease, the high cure rate associated with a low dose of pentamidine given for a short period makes it an attractive alternative to SSG and the treatment of choice in cases of fresh cases as well as SSG treatment failure cases (Berman, 1997).

1.1.4.6 **Cytokines**

Human recombinant interferon-? was used as an adjunct antimony therapy for visceral leishmaniasis (Badaro *et al.,* 1990). These investigators found that seven of nine cases of SSG resistant kala-azar could be cured with the combination of interferon ? given for 28 days. A subsequent trial showed that interferon ? was only partially effective by itself. It was found that interferon ?, in combination

with SSG, could speed the elimination of parasites in previously untreated patients (Sundar and Murray, 1995).

1.1.4.7 Allopurinol

Allopurinol was the first oral antileishmanial. This hypoxanthine analogue inhibits purine catabolism in mammalian cells and purine anabolism in *Leishmania*. It works on the principle that *Leishmania* species are unable to synthesize purines. Allopurinol is hydrolyzed to allopurinol riboside, an analogue of inosine that is incorporated instead of ATP into leishmanial RNA. There it interferes with the normal protein synthesis of the parasite (Singh and Sivakumar, 2004). Although allopurinol has been used to treat leishmaniasis for decades, a recent placebo-controlled double blinded trial in Colombian patients of cutaneous leishmaniasis caused by *L.panamensis* showed that allopurinol (20mg/kg/day for 28days) was no better than placebo. It was concluded that allopurinol monotherapy is ineffective against Colombian cutaneous disease and therefore is unlikely to be effective against other forms of leishmaniasis (Berman, 1997).

1.1.4.8 Imidazole derivatives

Metronidazole and other imidazole derivatives as well as several other oral drugs have been studied as antileishmanial agents (Mishra and Thakur, 1985). The principle quoted for using imidazoles as antileishmanial drugs is that the sterol composition of *Leishmania* species is similar to that of yeast and other fungi. Metronidazole eliminates only 30% of the parasites even when used at its peak

serum levels (30µg/ml) after intravenous administration. However, ketoconazole was found effective against *Leishmania* (Singh and Sivakumar, 2004). Hydrolyzed ketoconazole is the most effective drug that eliminated all parasites at a dose of 3.0 µg/ml and 80%–95% of the parasites at drug concentrations that are achievable. However, other derivatives such as miconazole and cotrimazole are found ineffective or toxic (Wali *et al.,* 1992). The effective dose of ketoconazole for cutaneous patients is 400mg/day for 28 days and for kala azar patients is 600 mg/day for 28 days.

The common side effect of ketoconazole is hepatotoxicity. Less commonly are endocrine dysfunction, reduction in cortisol levels and hypoadrenalism (Wali *et al.*, 1992).

1.1.4.9 Combination therapy

After increasing unresponsiveness to most of the monotherapeutic regimens, the combination therapy has found new scope in the treatment of both cutaneous and visceral leishmaniasis (Das *et al.,* 2001). Recently, combination therapy of sodium stibogluconate and indolylquinoline derivative A [2–2(2-dichloroacetamidobenzyle)-3-(39-indolylquinoline)] showed 100% clearance of the parasites from liver and spleen of the hamsters as compared to 93% and 80%, respectively, when indolylquinoline derivative A and SSG were used singly (Pal *et al.,* 2002). Similarly, the combination of low dosage pentamidine and

allopurinol was more effective in achieving an ultimate cure in 91.25% of kala-azar patients as compared to 74.35% using pentamidine alone (Becker *et al.*, 1999). All monotherapies of cutaneous leishmaniasis are less effective as compared to topical paromomycin plus methylbenzethonim chloride, curing 85.7%–91.4% cases. Other drugs tried are atovaquone, roxithromycin, and edelfosine. Drugs that have a long half-life and low therapeutic ratio, e.g., miltefosine, may induce drug resistance; therefore, such drugs should be used only in combination with another drug that has a short half life and greater therapeutic ratio (Bryceson, 2001).

1.1.5 Bioinformatics, the application of computational techniques to analyze the information associated with biomolecules on a large scale, has become an increasingly useful tool in molecular biology researches, both for genomic and proteomic analysis. In this study, the application of computational methods is used to identify sequence homology of drug target gene between leishmania species and other selected pathogens. This was feasible because of the accessibility of whole genome sequence and suitable software programs for homology search.

1.1.6 Leishmania genome

Leishmania species are diploid organism having a relatively small genome (approximately 34 Mb). Leishmania genome has high GC content (63%) , it contains 36 chromosomes pairs ranging in size from 0.3Mb to 2.5 Mb containing

about 8,000 predicted genes (Myler *et al.*, 2000). These genes are organized in tandom arrays or at least have two copies spreading through the genome.

The mitochondrial DNA of the kinetoplastida (kDNA) which constitutes 10%? 20% of the total DNA is mainly used as diagnostic tool to detect small amounts of parasite DNA in the biological material because of its highly repetitive nature (Barker, 1989).

1.1.7 Homology methods and sequence alignments

The bioinformatics identification of orthologos depends on sequence homology since similar sequence implies similar function. An array of bioinformatics techniques is available but I have selected basic local alignment search tool which is profile based algorithm for homologous sequence search. It finds regions of similarity between biological sequences, compares nucleotides or protein sequences to databases sequences and calculates the statistical significance of the matches. The net result was the selection of the following drugs:

Isoniazid

It is the most active drug for treatment of tuberculosis caused by susceptible strains. It acts by inhibiting the synthesis of mycolic acids, which are essential components of mycobacterial cell walls. It exerts its lethal effect by forming a covalent complex with an acyl carrier protein (AcpM) and KasA, a beta - ketoacyl carrier protein synthetase, which blocks mycolic acid synthesis.

Sulfadoxine and pyrimethamine combination

Sulfadoxine; hence is a sulfonamide; interferes with folic acid synthesis via inhibition of dihydrofolic acid formation from para-aminobenzoic acid. Pyrimethamine inhibits dihydrofolic acid reduction to tetrahydrofolate resulting in sequential inhibition of enzymes of folic acid pathway. This combination is widely used as second line treatment of malaria.

1.2 RATIONALE

Most of current antileishmanial agents are toxic, expensive, given by injection; require hospitalization for long duration with evidence of emergence of parasite resistance to several antieshmanial drugs. The successful completion of the leishmania whole genome sequencing provide an excellent opportunity for identification of new drug targets for production of safe, effective and affordable drugs. The fact that drugs used for treatment of some diseases are effective against unrelated pathogen is encouraging to search of similar findings.

1.3 OBJECTIVES

General objectives:

To identify and evaluate selected drugs used for treatment of other diseases for antileishmanial activity using comparative bioinformatics.

Specific objectives:

1-To determine the DNA and / or protein sequence homology of known pathogens targeted by known drugs with the sequence of the Leishmania species.

2-To evaluate the sensitivity of the selected drugs against leishmania using in vitro drug sensitivity assay.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Parasites

Leishmania donovani (institute of endemic diseases)

Leishmania major (institute of endemic diseases)

Macrophage cell line

THP1 (institute of endemic diseases)

Media

NNN agar (Sigma Aldrich; Germany)

DMEM (Sigma Aldrich; Germany)

Drugs

Isoniazid (Sigma Aldrich; Germany)

AmBisome (Gilead science LTD; Ireland)

Sodium stibogluconate (Albert David LTD; India)

Sulfadoxine pyrimethamine combination (Efroze chemical industries; Pakistan)

2.2 Methods

2.2.1 Selection of candidate drugs

Isoniazid and combination of sulfadoxine and pyrimethbamine were selected based on their mode of action on mycobacterium tuberculosis and plasmodium parasite respectively.

2.2.3 Identification of pathogen target sequence for the selected drug

Isoniazid inhibits the enzyme fatty acid synthase of the mycobacterium, sulfadoxine inhibits the enzyme ptreoate synthase and pyrimethamine inhibits the enzyme dihydrofolate reductase of the plasmodium. The protein sequence of these enzymes was retrieved from the database (protein database of ncbi.nlm.nih.gov).

2.2.4 Alignment of the pathogen target sequence with leishmania sequence

Leishmania sequence of these enzymes was retrieved from the database (protein database of ncbi.nlm.nih.gov) and aligned with the pathogen target sequence. Homology between leishmania sequence and pathogen sequence was determined using basic local search alignment tool (BLAST).

2.2.4 Evaluation of the antileishmanial activity of the candidate drugs on leishmania using THP1 macrophage cell line

Two leishmania species were used in this study *L.donovani* which was isolated from lymph node aspirate of VL patient from Gadaref state and *L.major* which was isolated from skin lesion of CL patient from Khartoum state. The promastigotes of the two species were used for in vitro THP1 macrophage cell line infection.

2.2.4.1 Parasite isolation and culture

Leishmania parasites were isolated and incubated at 26°c in NNN agar tubes. Three days after incubation, smears were prepared to ensure the growth and check of contamnation. The primary cultures were sub cultured in freshly prepared biphasic media.

When parasite growth was established, the promastigotes were sub cultured in a sterile DMEM media supplemented with 10% heat inactivated fetal calf serum and 1% antibiotics (pencillin5000 IU/ml and streptomycin 5mg/ml). The cultures were incubated at 26°c. The cultures were examined using the inverted microscope to monitor growth and contamination every 2 days. Growing leishmania promastigotes were sub cultured in complete media and incubated at 26°c for 7 days. Under these culture conditions the stationary growth phase was

reached. Giemsa stained smears were prepared and examined to check for the purity of the culture.

2.2.4.2 Molecular characterization of the isolates

2.2.4.2.1DNA extraction using Chelex method

Growing promatigotes were harvested by centrifugation at 13000 rpm for 5 minutes; the pellets were used for DNA extraction.

Two hundred micro liters (200 µL) of well mixed 5%(W/V) chelex-100 slurry was transferred into two sterile micro centrifuge tubes 100 µL in each, which were heated at 100°C for 5 minutes by placing in the heating block of PERKIN ELMER DNA thermal cycler (480 USA). Hundred micro liters (100 µL) of *L.donovani* pellets was added into one tube and 100 µL of *L .major* pellets was added into the second tube. The mixtures were further heated at 100°C for 10 minutes, and then centrifuged at 13000 rpm for 3 minutes. The upper layer was transferred into new tubes for polymerase chain reaction.

2.2.4.2.2Polymerase chain reaction (PCR)

Kinetoplast DNA (KDNA-PCR)

KDNA-PCR was done using genus specific primers for mini circle kinetoplast DNA (kDNA) the AJS3, 5'ggggTTggTgTAAAATAgggC-3' and DBY 5'CCAGTTTCCCgCCCCggAg-3' primers. The reaction volume was 50µL per

sample in 0.5 ml thin walled micro centrifuge tube. The mixture consisted of 2.5 µL 10x reaction buffer, 1.5 µL of 20mM dNTPs mix (ABgene-0196-UK), 2.0 µL of 25 mM $MgCl_2$, 2.0 µL of each primer (10 mM each) and 0.3 µL of thermo stable DNA polymerase (5U/ µL). For each tube of PCR, 4 µL of template DNA were added; PCR mixture was completed to 25 µL with double distilled water. The reaction mixture was overlaid with 25 µL mineral oil. PCR was done using advanced primus (PEQ lab) PCR machine. The amplification was done for 35 cycles. The DNA was initially denatured at 94 °C for 3 minutes. Each cycle included annealing at 64°C for 1 minute, extension at 72 °C for 1 minute and denaturation at 94°C for 3 seconds. A final extension cycle at 72°C for 10 minutes was run. The PCR products were analyzed for quantity and quality using the nano drop spectrophotometer and stored at 4 °C until analyzed by agarose gel electrophoresis.

2.2.4.2.3PCR product analysis

Agarose gel electrophoresis

1.5% agarose gel was prepared by dissolving 1.5 gm of agarose in 100 ml 10% Tris Boric EDTA (TBE) with heating. After the dissolution of the agarose, 3 µL of 10 mg/ml ethidium bromide was added to the gel and mixed. The mixture was poured into horizontal electrophoresis mini gel tank with a suitable size combs and the gel was left for 1 hour to polymerize. 7 µL of PCR product were mixed

with 7 µL of the loading buffer and loaded into the wells. 5 µL of DNA molecular weight marker was loaded into a separate well. Running buffer which consists of 1.5 ml 10% TBE and 250 ml distilled water was added. Electrophoresis was run for 1 hour at 70 volt and the current ranges from 3 to 5. The gel was visualized over ultra violet transilluminater (ANACHEM UK) and photographed using gel documentation system (SYNGENE BIOIMAGING system).

2.2.4.3Media preparation

2.2.4.3.1 Preparation of NNN media

The modified NNN media consisted of solid and liquid phase. The solid phase was prepared by dissolving 2.5 gm blood agar base in 100 ml distilled water. The mixture was then boiled, autoclaved and cooled to 50°c. 10 ml of rabbit blood was taken and into tube containing glass beads then it was shacked for 15 minutes to remove the fibrinogen. The defibrinated blood was added to the mixture. 1% of Antibiotic mixture consisting of 5000 IU/ml penicillin and 5 mg/ml streptomycin was added. Then the media was liquated into sterile glass tubes that were laid in a slope position till the agar solidified. The tubes were incubated at 4°c for 24 hours and the liquid phase consisting of 10% fetal calf serum and 90% CM was added to the media just before use.

2.2.4.3.2 Preparation of the complete media (CM)

17.3 gm Dulbecco's modified eagle's medium (DMEM) with L- glutamine, 4500 mg glucose and 25 Mm HEPES PH 7.4 was dissolved in 1L distilled water using magnetic shaker. Then 3.7 gm $NaHCO_3$ was added and the pH was measured using pH meter and adjusted to 6.1? 6.3 using 1N NaOH or 1N HCL, the media was filtered using filter membrane of 0.22 µ porosity. 10% heat inactivated fetal calf serum and 1% antibiotic mixture (5000 IU/ml penicillin and 5 mg/ml streptomycin) were added.

2.2.4.3.3 Preparation of the freezing media

10 ml of sterile glycerol, 30 ml of Dulbecco's modified eagle's medium (DMEM) and 60 ml of heat inactivated fetal calf serum were mixed together to produce 100 ml freezing media.

2.2.4.4 Preparation of the stains

2.2.4.4.1 Preparation of Giemsa stain stock solution

Giemsa's solution is a mixture of methylene blue, eosin and azure B. The stain was prepared by dissolving 3.8g of Giemsa powder in 250 ml methanol, the solution was heated to 60°C and then slowly 250 ml glycerin was added and the solution was filtered and allowed to stand.

2.2.4.4.2 Preparation of Giemsa stain working solution

Working solution was prepared by adding 90 ml distilled water to 10 ml stock solution.

2.2.4.5 In vitro macrophage cell line culture

Human macrophage (THP1) cell line were cultured in sterile DMEM complete media in disposable tissue culture flasks Under sterile condition the contents of the thawed culture tube was transferred into 15 ml sterile falcon tube and 2mL of complete media was added to wash the cells from dimethylsulphoxide (DMSO) or glycerol in the freezing media. The cells were centrifuged at 1200 rpm for 10 minutes at 4°c. The supernatant was discarded and 1mL of complete media was added to the pellets and mixed well. The cells were transferred into 50 mL sterile tissue culture flask and about 10-15 ml of complete media was added and incubated in 5% CO_2 atmosphere at 37°c. The cells were examined under the inverted microscope every day to detect growth and contamination.

2.2.4.6 Cell freezing

Well growing macrophage culture was centrifuged at 1300 rpm for 10 minutes. The supernatant was discarded and the pellets were resuspended in 0.5 ml complete media. 300 µl was taken into cryovial tube and the volume was completed to 1 ml using the freezing media. Tubes were stored in -80°C refrigerator as stock.

2.2.4.7 In vitro infection of the macrophage with the leishmania parasite

Cultures of the stationary growth phase of the parasite were transferred into 15 ml sterile falcon tubes and centrifuged at 1200 rpm for 10 minutes; the pellets were resuspended in 1 ml complete media. 10 µL was taken and mixed with 90 µL of formalin 2%. 5 µL of the mixture was used for counting of the promastigotes using haemocytometer.

Cultures of well grown macrophages were transferred into 15 ml falcon tube and centrifuged at 1200 rpm for 10 minutes; the pellets were resuspended in 1 ml complete media. 10 µL was added to 90µL of trypan blue 0.4% for counting of live macrophages using haemocytometer. The growing THP1 cells were infected by stationary phase promastigotes of *L. donovani* and *L.major* at ratio 1:5 in sterile tissue culture plates. The infected culture was incubated in 5% CO_2 atmosphere at 37°c. The drug was added 3 days post infection.

2.2.4.8 Measurement of the parasite inhibition and drug assay

2.2.4.8.1 Preparations of the drugs

Isoniazid:

Isoniazid was obtained from Sigma Aldrich (Germany) as standard powder of known potency. Five (5) mg of powder was dissolved in 5mL distilled water to produce a solution of 1 mg/ml (1µg/µl) concentration. The solution was filtered

using a membrane filter of 0.22 porosity. Doses of volume equivalent to 0.1, 0.2 and 0.4 µg/ml were prepared using sterile water for injection.

Sulfadoxine and pyrimethamine combination:

The combination was obtained as tablets from Efroze chemical industries (Pakistan). 2 tablets were ground, dissolved in sterile water for injection and filtered by using a membrane filter of 0.22 porosity. Doses of volume equivalent to 1.9, 2.9, 3.8 µg/ml pyrimethamine and 38.1, 57.1, 76.2 µg/ml sulphadoxine was prepared.

Sodium stibogluconate

It was provided as intravenous solution equivalent to 100 mg/ml pentavalent antimony from Albert David LTD (India). Serial dilutions were made to prepare doses of 20µg/ml for *L. major* and 24µg/ml for *L. donovani*.

Liposomal amphotericin B

Ambisome was provided as 50 mg powder for concentrate for IV infusion from Gilead sciences LTD (Ireland). Serial dilutions were made to prepare dose of 10µg/ml for *L. major* and *L. donovani*.

2.2.4.8.2 The effect on the promastigotes

Growth inhibition of different drug concentrations was determined by comparing

the activity of treated promastigotes culture with untreated and SSG treated cultures. This was done by examining the cultures under the inverted microscope after 1, 2, 3, 24, 48 and 72 hrs.

2.2.4.8.3 The effect on the amastigotes

Three days post addition of the drug the treated and untreated macrophage infected cultures were centrifuged at 12000 rpm for 10 minutes, the supernatant was discarded and the pellets were resuspended in 1ml complete media, then smears were prepared and examined under the microscope. Percentage of killing for each drug was calculated.

2.2.4.8.4 Isoniazid (INH) experiments

The effect on the promastigotes

L.major **promastigotes:** 1000 µl of growing *L.major* culture was added into 15 well of 24 wells tissue culture plate. The drug was added at 0.1, 0.2 and 0.4 µg/ml in triplicate wells. Promastigote culture without Isonaizid and Promastigote culture with 20µg/ml SSG were added in triplicate wells and used as controls.

L.donovani **promastigotes:** 1000 µl of well growing *L.donovani* culture was added into 15 well of 24 wells tissue culture plate. The drug was added at 0.1, 0.2 and 0.4 µg/ml in triplicate wells. Promastigote culture without Isonaizid and Promastigote culture with 24µg/ml SSG were added in triplicate wells and used as controls.

The effect on the amastigotes

L.major **amastigotes**: 1000 µl of *L. major* infected macrophage culture was added into 15 well of 24 wells tissue culture plate. The drug was added at 0.1, 0.2 and 0.4 µg/ml in triplicate wells. Infected macrophages without Isonaizid and infected macrophages with 20µg/ml SSG were added in triplicate wells and used as controls.

L.donovani **amastigotes***:* 1000 µl of *L.donovani* infected macrophage culture was added into 15 well of 24 wells tissue culture plate. The drug was added at 0.1, 0.2

and 0.4 µg/ml in triplicate wells. Infected macrophages without Isonaizid and infected macrophages with 24µg/ml SSG were added in triplicate wells and used as controls.

2.2.4.8.5 Combination of INH and SSG experiments

The effect on the promastigotes

L.major **promastigotes:** 1000 µl of growing *L.major* culture was added into 24 well of 24 wells tissue culture plate. The drugs were added at 0.1, 0.2 and 0.4 µg/ml of INH and 0.1, 0.2 and 0.4 each with 20 µg/ml SSG in triplicate wells. Promastigote culture without the drugs and promastigote culture with 20µg/ml SSG were added in triplicate wells and used as controls.

L. donovani **promastigotes:** 1000 µl of growing *L.donovani* culture was added into 24 well of 24 wells tissue culture plate. The drugs were added at 0.1, 0.2 and 0.4 µg/ml of INH and 0.1, 0.2 and 0.4 each with 24 µg/ml SSG in triplicate wells. Promastigote culture without the drugs and promastigote culture with 24µg/ml SSG were added in triplicate wells and used as controls.

The effect on the amastigotes

L.major **amastigotes:** 1000 µl of *L. major* infected macrophage culture was added into 24 well of 24 wells tissue culture plate. The drug was added at 0.1, 0.2 and 0.4 µg/ml of INH and 0.1, 0.2 and 0.4 each with 20 µg/ml SSG in triplicate

wells. Infected macrophages without the drugs and infected macrophages with 20µg/ml SSG were added in triplicate wells and used as controls.

L.donovani **amastigotes**: 1000 µl of *L.donovani* infected macrophage culture was added into 24 well of 24 wells tissue culture plate. The drug was added at 0.1, 0.2 and 0.4 µg/ml of INH and 0.1, 0.2 and 0.4 each with 24 µg/ml SSG in triplicate wells. Infected macrophages without the drugs and infected macrophages with 24µg/ml SSG were added in triplicate wells and used as controls.

2.2.4.8.6 Combination of INH and ambisome experiment

The effect on the promastigotes

L.major **promastigotes:** 1000 µl of growing *L.major* culture was added into 24 well of 24 wells tissue culture plate. The drugs were added at 0.1, 0.2 and 0.4 µg/ml of INH and 0.1, 0.2 and 0.4 each with 10 µg/ml amBisome in triplicate wells. Promastigote culture without the drugs and Promastigote culture with 10µg/ml amBisome were added in triplicate wells and used as controls.

L.donovani **promastigotes:** 1000 µl of growing *L.donovani* culture was added into 24 well of 24 wells tissue culture plate. The drugs were added at 0.1, 0.2 and 0.4 µg/ml of INH and 0.1, 0.2 and 0.4 each with 10µg/ml amBisome in triplicate wells. Promastigote culture without the drugs and Promastigote culture with 10µg/ml amBisome were added in triplicate wells and used as controls.

The effect on the amastigotes

L.major **amastigotes:** 1000 µl of *L. major* infected macrophage culture was added into 24 well of 24 wells tissue culture plate. The drug was added at 0.1, 0.2 and 0.4 µg/ml of INH and 0.1, 0.2 and 0.4 each with 10µg/ml amBisome in triplicate wells. Infected macrophages without the drugs and infected macrophages with 10µg/ml amBisome were added in triplicate wells and used as controls.

L.donovani **amastigotes**: 1000 µl of *L.donovani* infected macrophage culture was added into 24 well of 24 wells tissue culture plate. The drug was added at 0.1, 0.2 and 0.4 µg/ml of INH and 0.1, 0.2 and 0.4 each with 10µg/ml amBisome in triplicate wells. Infected macrophages without the drugs and infected macrophages with 10µg/ml amBisome were used as controls.

2.2.4.8.7 Combination of sulfadoxine and pyrimethamine experiment

Table (1): Concentration of sulphadoxine ? pyrimethamine Combination.

	Concentration (µg/ml)
1	1.9 pyrimethamine and 38.1 sulfadoxine
2	2.9 pyrimethamine and 57.1sulfadoxine
3	3.8 pyrimethamine and 76.2sulfadoxine

The effect on the promastigotes

L.major **promastigotes:** 1000 µl of growing *L.major* culture was added into 15 well of 24 wells tissue culture plate. The drug was added at the three different concentrations in triplicate wells. Promastigote culture without the drug and promastigote culture with 20µg/ml SSG were added in triplicate wells and used as controls.

L.donovani **promastigotes:** 1000 µl of growing *L.donovani* culture was added into 15 well of 24 wells tissue culture plate. The drug was added at the three different concentrations in triplicate wells. Promastigote culture without the drug

and promastigote culture with 24µg/ml SSG were added in triplicate wells and used as controls.

The effect on the amastigotes

L.major **amastigotes:** 1000 µl of *L. major* infected macrophage culture was added into 15 well of 24 wells tissue culture plate. The drug was added at the three different concentrations in triplicate wells. Infected macrophages without the drug and infected macrophages with 20µg/ml SSG were added in triplicate wells and used as controls.

L.donovani **amastigotes**: 1000 µl of *L.donovani* infected macrophage culture was added into 15 well of 24 wells tissue culture plate. The drug was added at the three different concentrations in triplicate wells. Infected macrophages without the drug and infected macrophages with 24µg/ml SSG were added in triplicate wells and used as controls.

2.2.4.9 Preparation of Geimsa stained smears

30 µL of the infected culture was taken; air dried and fixed with 70% methanol and stained with 10% Geimsa stain for 10 minutes and rinsed with tap water, then examined under the microscope.

2.2.5 Statistical analysis

The results were statistically analyzed using paired sample student t test taking 0.05 as level of significance.

Chapter three

Results

3.1 Alignment of the pathogen target sequence and leishmania sequence

3.1.1 Alignment of leishmania major 3-oxoacyl- acyl carrier protein synthaseII and mycobacterium fatty acid synthase

Query: 3-oxoacyl-acyl carrier protein synthase ii; beta-ketoacyl synthase family protein (Leishmania major >gi|68129107|emb|CAJ06755.1| putative beta-ketoacyl synthase family protein (Leishmania major strain Friedlin)

Subject: fatty acid synthase (Mycobacterium tuberculosis H37Rv) >gi|148662361|ref|YP_001283884.1

Table (2): Alignment score of isoniazid target sequence in *L.major* and *M. tuberculosis.*

Max score	Total score	Query coverage	E value
55.5	134	64%	3e? 11

Dot matrix view

Plot of leishmania major protein sequence vs mycobacterium protein sequence

This dot matrix view shows regions of similarity based upon the BLAST results. The query sequence is represented on the X-axis and the subject is represented on

the Y-axis. The numbers represent the bases/residues of the subject. Alignments are shown in the plot as lines. Figure (9).

3.1.2 Alignment of leishmania donovani 3-oxoacyl- acyl carrier protein synthaseII and mycobacterium fatty acid synthase

Query: unnamed protein product (Leishmania donovani BPK282A1) gi|322502344|emb|CBZ37428.1|.

Subject: fatty acid synthase (Mycobacterium tuberculosis H37Rv) >gi|148662361|ref|YP_001283884.1

Table (3): Alignment score of Isoniazid target sequence in *L.donovani* and *M. tuberculosis*

Max score	Total score	Query coverage	E value
54.7	132	57%	5e? 11

Dot matrix view

Plot of leishmania donovani protein sequence vs mycobacterium protein sequence

This dot matrix view shows regions of similarity based upon the BLAST results. The query sequence is represented on the X-axis and the numbers represent the

bases/residues of the query. The subject is represented on the Y-axis and again the numbers represent the bases/residues of the subject. Alignments are shown in the plot as lines. Figure (10).

3.1.3 Alignment of dihydrofolate reductase-thymidylate synthase (Leishmania major) and dihydrofolate reductase-thymidylate synthase (Plasmodium falciparum)

Query: dihydrofolate reductase-thymidylate synthase (Leishmania major) >gi|118936|sp|P07382.1|.

Subject: dihydrofolate reductase-thymidylate synthase (Plasmodium falciparum) >gi|623444|gb|AAB59212.1|.

Table (4): Alignment score of pyrimethamine? sulfadoxine target sequence in *L.major* and *P. falciparum.*

Max score	Total score	Query coverage	E value
341	454	91%	4e? 98

Dot matrix view

Plot of leishmania protein sequence vs. plasmodium sequence

This dot matrix view shows regions of similarity based upon the BLAST results.

The query sequence is represented on the X-axis and the numbers represent the bases/residues of the query. The subject is represented on the Y-axis and again the numbers represent the bases/residues of the subject. Alignments are shown in the plot as lines. Figure (11).

3.1.4 Alignment of dihydrofolate reductase-thymidylate synthase (Leishmania major) and dihydrofolate reductase-thymidylate synthase (Plasmodium vivax)

Query: dihydrofolate reductase-thymidylate synthase (Leishmania major) >gi|118936|sp|P07382.1|.

Subject: dihydrofolate reductase (Plasmodium vivax) gi|3550616|emb|CAA05916.1|.

Table (5): Alignment score of pyrimethamine? sulfadoxine target sequence in *L.major* and *P. vivax*

Max score	Total score	Query coverage	E value
65.1	65.1	30%	2e? 15

Dot matrix view

Plot of leishmania protein sequence vs. plasmodium sequence

This dot matrix view shows regions of similarity based upon the BLAST results.

The query sequence is represented on the X-axis and the numbers represent the bases/residues of the query. The subject is represented on the Y-axis and again the numbers represent the bases/residues of the subject. Alignments are shown in the plot as lines. Figure (12).

3.1.5 Alignment of dihydrofolate reductase-thymidylate synthase (Leishmania donovani) and dihydrofolate reductase-thymidylate synthase (Plasmodium falciparum)

Query: dihydrofolate reductase-thymidylate synthase (Leishmania donovani) gi|322496602|emb|CBZ31672.1|

Subject: dihydrofolate reductase-thymidylate synthase (Plasmodium falciparum) gi|623444|gb|AAB59212.1|

Table (6): Alignment score of pyrimethamine? sulfadoxine target sequence in *L.donovani* and *P. falciparum.*

Max score	Total score	Query coverage	E value
340	452	91%	1e? 97

Dot matrix view

Plot of leishmania protein sequence vs. plasmodium sequence

This dot matrix view shows regions of similarity based upon the BLAST results.

The query sequence is represented on the X-axis and the numbers represent the bases/residues of the query. The subject is represented on the Y-axis and again the numbers represent the bases/residues of the subject. Alignments are shown in the plot as lines. Figure (13).

3.1.6 Alignment of dihydrofolate reductase-thymidylate synthase (Leishmania donovani) and dihydrofolate reductase-thymidylate synthase (Plasmodium vivax)

Query: dihydrofolate reductase-thymidylate synthase [Leishmania donovani] gi|322496602|emb|CBZ31672.1|

Subject: dihydrofolate reductase-thymidylate synthase [Plasmodium vivax] gi|99082919|gb|ABF66633.1|

Table (7): Alignment score of pyrimethamine? sulfadoxine target sequence in *L.donovani* and *P. vivax.*

Max score	Total score	Query coverage	E value
332	399	85%	3e? 95

Dot matrix view

Plot of leishmania protein sequence vs. plasmodium sequence

This dot matrix view shows regions of similarity based upon the BLAST results.

The query sequence is represented on the X-axis and the numbers represent the bases/residues of the query. The subject is represented on the Y-axis and again the numbers represent the bases/residues of the subject. Alignments are shown in the plot as lines. Figure (14).

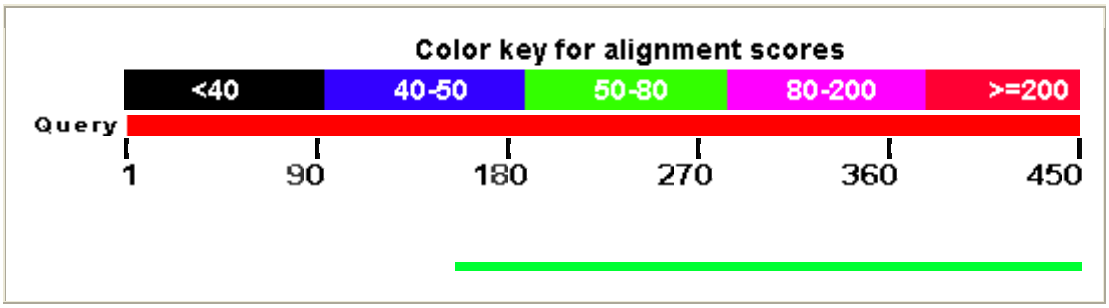

Figure (3): Alignment score of isoniazid target sequence in *L.major* and *M. tuberculosis.*

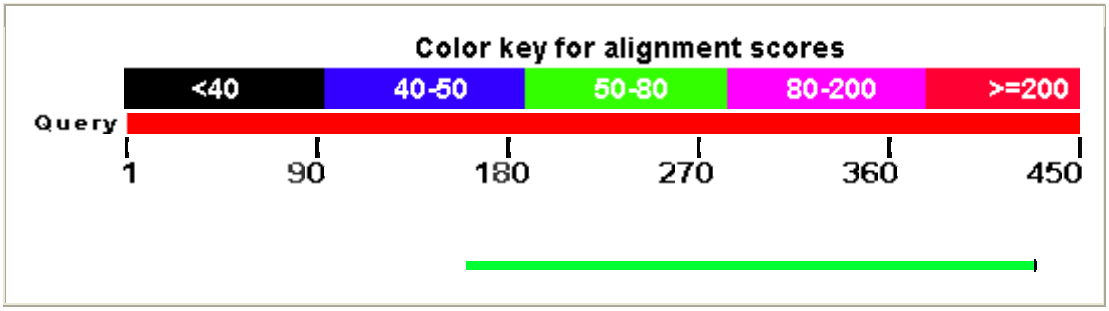

Figure (4): Alignment score of Isoniazid target sequence in *L.donovani* and *M. tuberculosis.*

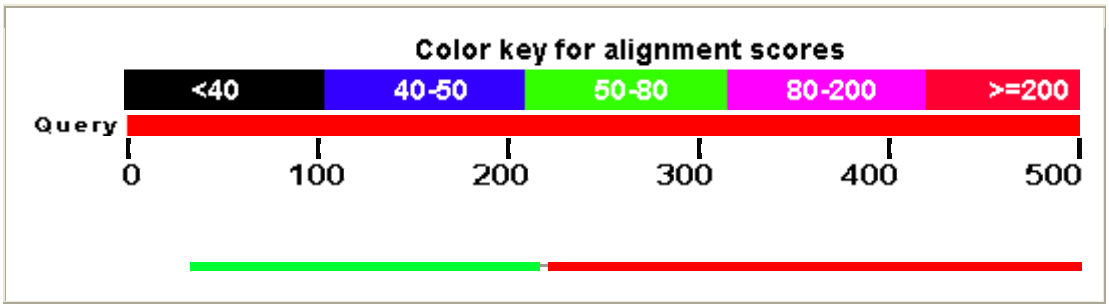

Figure (5): Alignment score of pyrimethamine? sulfadoxine target sequence in *L.major* and *P. falciparum.*

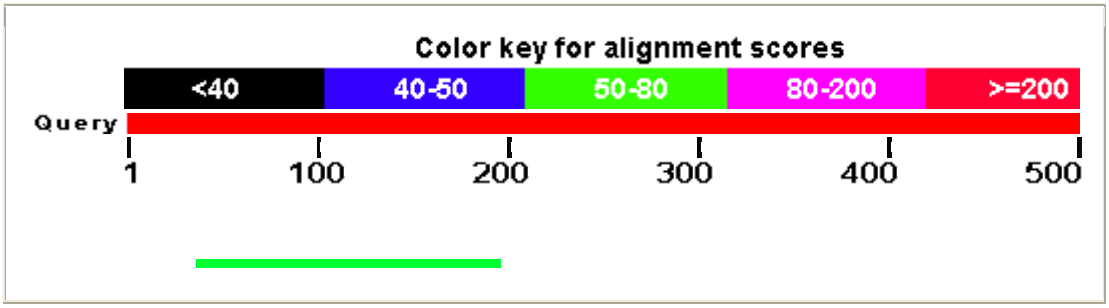

Figure (6): Alignment score of pyrimethamine? sulfadoxine target sequence in *L.major* and *P. vivax.*

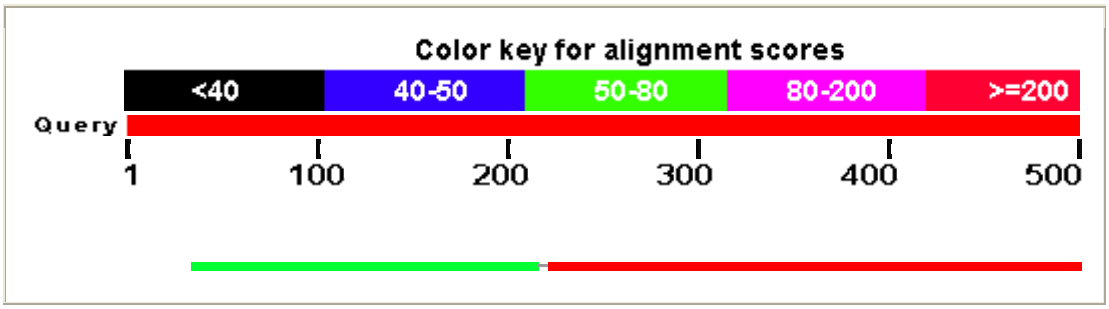

Figure (7): Alignment score of pyrimethamine? sulfadoxine target sequence in *L.donovani* and *P. falciparum.*

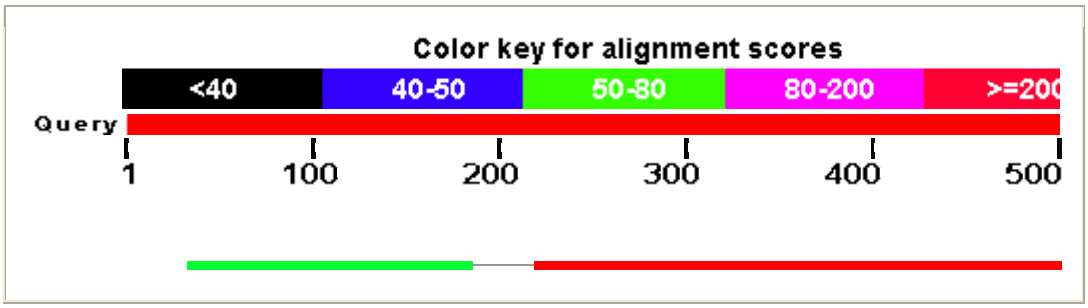

Figure (8): Alignment score of pyrimethamine? sulfadoxine target sequence in *L.donovani* and *P. vivax.*

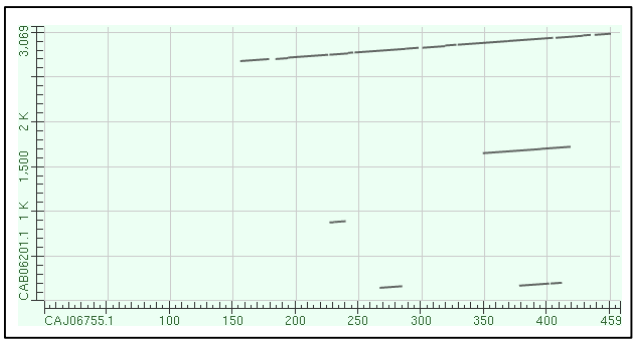

Fig (9): Dot matrix view of blasting 3-oxoacyl-acyl carrier protein synthase ii (Leishmania major strain Friedlin) and fatty acid synthase (Mycobacterium tuberculosis H37Rv]).

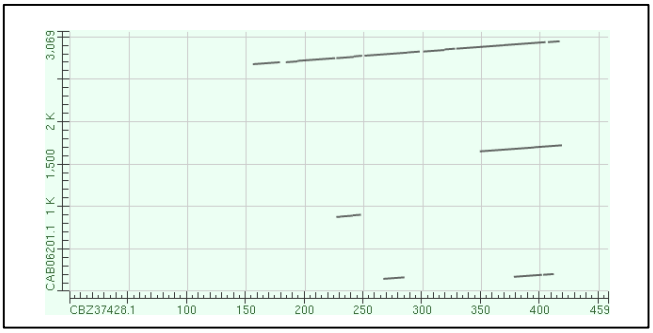

Fig (10): Dot matrix view of blasting unnamed protein product (Leishmania donovani) and Fatty acid synthase (Mycobacterium tuberculosis H37Rv).

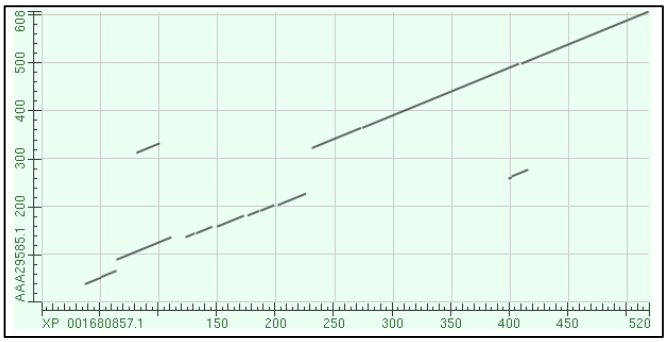

Fig (11): Dot matrix view of blasting dihydrofolate reductase-thymidylate synthase (Leishmania major) and dihydrofolate reductase-thymidylate synthase (Plasmodium falciparum).

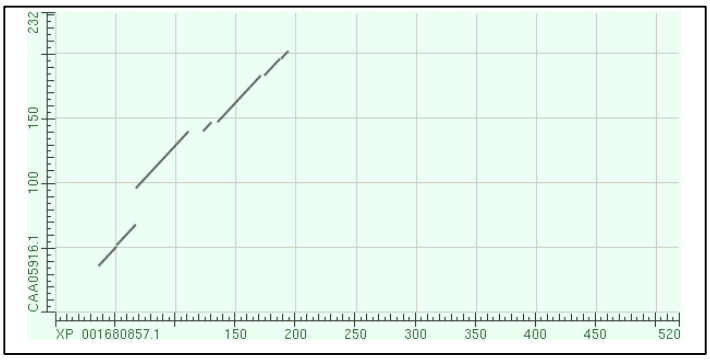

Fig (12): Dot matrix view of blasting dihydrofolate reductase-thymidylate synthase (Leishmania major) and dihydrofolate reductase-thymidylate synthase (Plasmodium vivax).

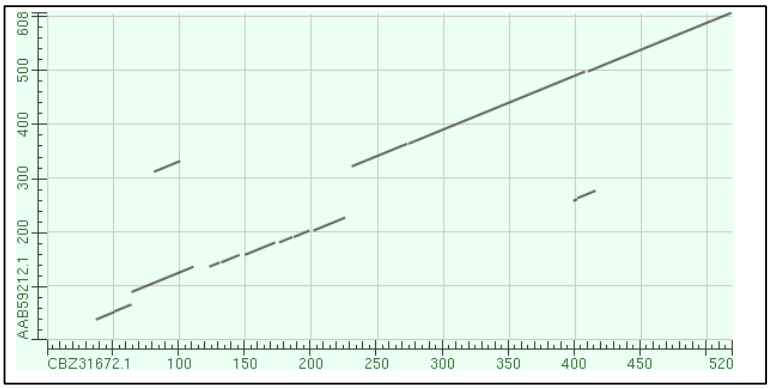

Fig (13): Dot matrix view of blasting dihydrofolate reductase-thymidylate synthase (Leishmania donovani) and dihydrofolate reductase-thymidylate synthase (Plasmodium falciparum).

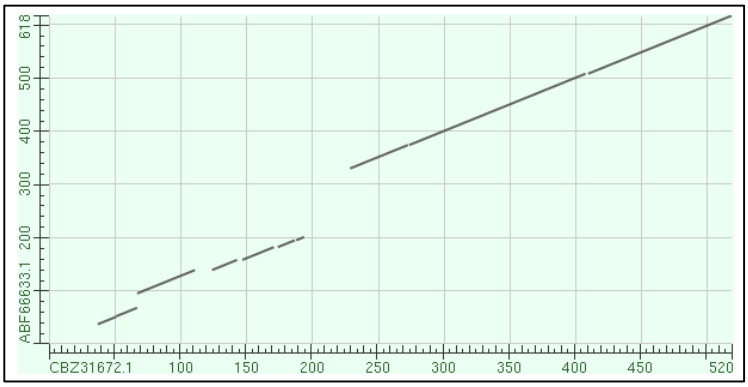

Fig (14): Dot matrix view of blasting dihydrofolate reductase-thymidylate synthase (Leishmania donovani) and dihydrofolate reductase-thymidylate synthase(Plasmodium vivax).

3.2 Evaluation of the antileishmanial activity of the candidate drugs on leishmania using THP1 macrophage cell line

3.2.1 Parasite isolates and culture

The growth of leishmania parasite promastigotes was achieved in both NNN and CM. figures (32 and 33).

3.2.2 Molecular characterization of the isolate

Kinetoplast DNA (KDNA-PCR)

Based on the size of the DNA amplified band, *L.donovani* complex showed an amplicon of 800 bp, and *L.* major showed an amplicon of 700 bp figure (15).

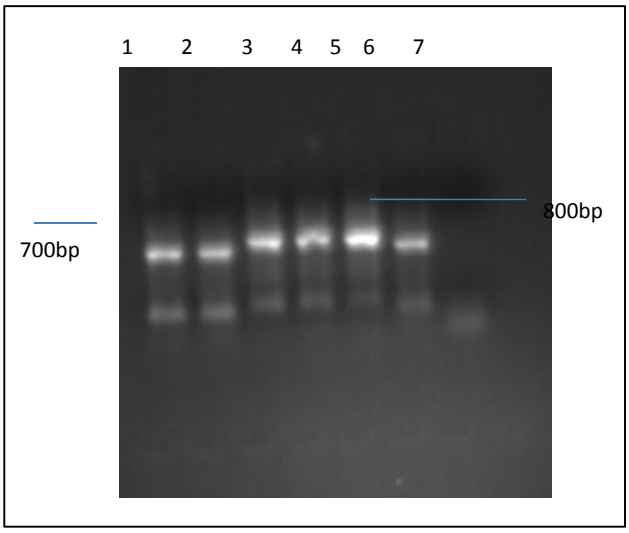

Fig (15): Stained agarose gel electrophoresis of PCR amplified kDNA of leishmania. Lane 1, 2: *L.major*, lane 3, 4: L.*donovani*, lane 5: positive control *L.donovani*, lane 6: positive control *L.major*, lane7: negative control.

3.2.3 Measurement of the parasite inhibition and drug assay

3.2.3.1 The effect on the promastigotes

1- INH

There was no detectable effect in the activity in all wells of *L.major* and *L.donovani* after 1, 2 and 3 hrs. But after 24 hrs, there was a lot of clusters with round shape formation as compared to SSG treated and negative control. After 48 hrs almost 90% of the promastigotes have transformed to round shaped. After 72 hrs no change was observed.

2- INH plus SSG

The results were similar to that of INH alone

3- INH plus amBisome

There was no detectable effect in the activity in all wells of *L.major* and *L.donovani* after 1and 2hrs. However after 3 hrs, there was marked reduction in the activity, and a complete reduction of the activity was detected after 24 hrs and 48 hrs compared with the negative control.

4- Sulphadoxine- pyrimethamine combination

There was no reduction in the activity in all wells of *L.major* and *L.donovani* after 1, 2 and 3 hrs. After 24 hrs of incubation, a change in the shape, activity

and clustering of the parasites was detected in the treatment wells compared with SSG and the negative controls. After 48 and 72 hrs there was marked activity reduction.

3.2.3.2 The effect on the amastigotes

3.2.3.2.1 Antileishmanial activity of isoniazid against *L.donovani* using in vitro macrophage infection assay

To evaluate the antileishmanial activity of INH against *leishmania donovani* amastigotes, the following concentrations of INH were added to macrophage infected cells, 0.1, 0.2 and 0.4µg/ml. The results showed a decrease in the mean number of amastigotes per 100 THP1 as compared to the untreated cells (negative control) and increase in the percentage of killing as shown in figure (16 and 17).

Table (8): Mean number of *leishmania donovani* amastigotes per 100 THP1 and percentage of killing produced by 0.1, 0.2 and 0.4 µg/ml of isoniazid and 24 µg/ml of SSG.

	Concentration				**Negative control**
	0.1	0.2	0.4	24 SSG	
Mean No of amastigotes Per 100THP1 ± SEM	374* ±8.5	362* ±10.7	332* ±8.5	277* ±22.5	792 ±13.2
% of killing	52.8%	54.3%	58.8%	65%	–

Note: * indicates significance level less than 0.05 ($P < 0.05$).

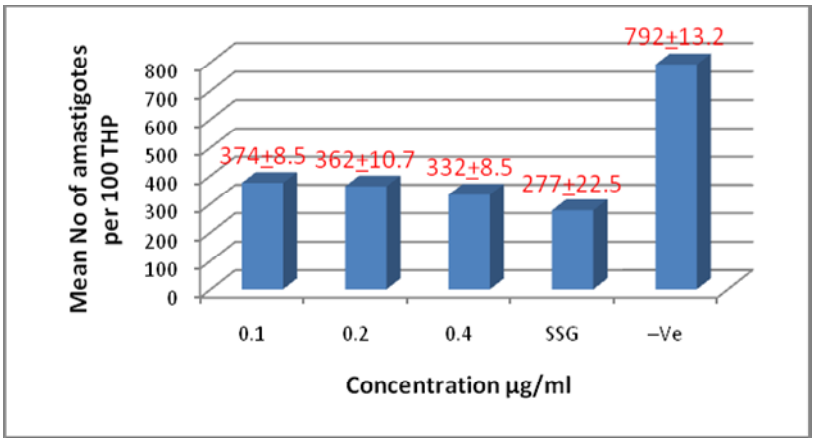

Fig (16): Mean number *Leishmania donovani* amastigotes per 100 THP1 in the presence of isoniazid using in vitro THP1 infection assay.

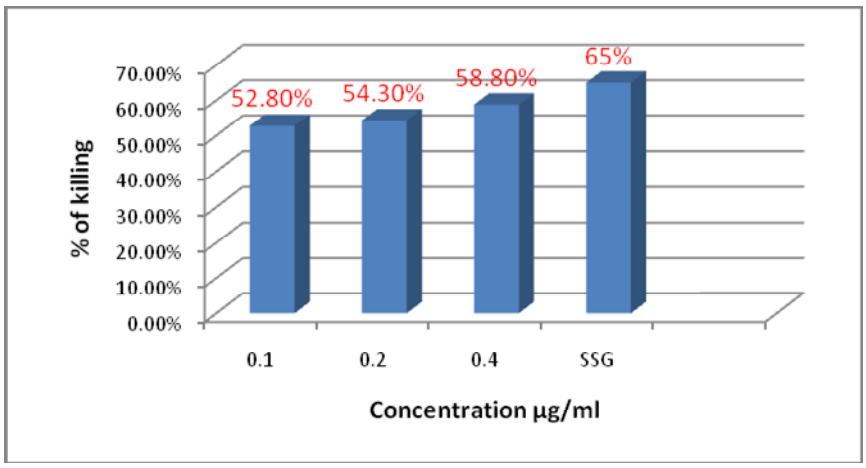

Fig (17): Percentage of killing of *Leishmania donovani* amastigotes in the presence of isoniazid using in vitro THP1 infection assay.

3.2.3.2.2 Antileishmanial activity of isoniazid against *L.major* using in vitro macrophage infection assay

To evaluate the antileishmanial activity of INH against *leishmania major* amastigotes, the following concentrations of INH were added to macrophage infected cells, 0.1, 0.2 and 0.4µg/ml. The results showed a decrease in the mean number of amastigotes per 100 THP1 as compared to the untreated cells (negative control) and increase in the percentage of killing as shown in figure (18 and 19).

Table (9): Mean number of *leishmania major* amastigotes per 100 THP1 and percentage of killing produced by 0.1, 0.2 and 0.4 µg/ml of isoniazid and 20 µg/ml of SSG.

	Concentration				**Negative control**
	0.1	0.2	0.4	20 SSG	
Mean No of amastigotes Per 100THP1 ±SEM	542*±47.1	459*±48.3	313*±21.4	333*±10.7	785±38.7
% of killing	31%	41.5%	60.2%	57.6%	–

Note: * indicates significance level less than 0.05 ($P < 0.05$).

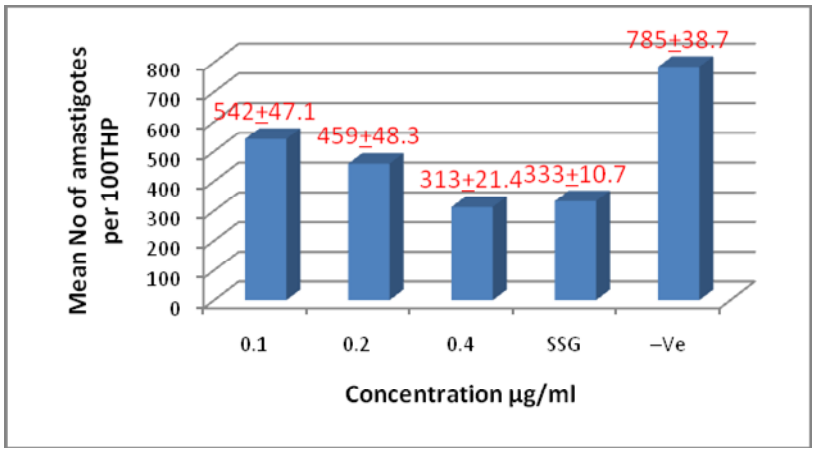

Fig (18): Mean number *Leishmania major* amastigotes per 100 THP1 in the presence of isoniazid using in vitro THP1 infection assay.

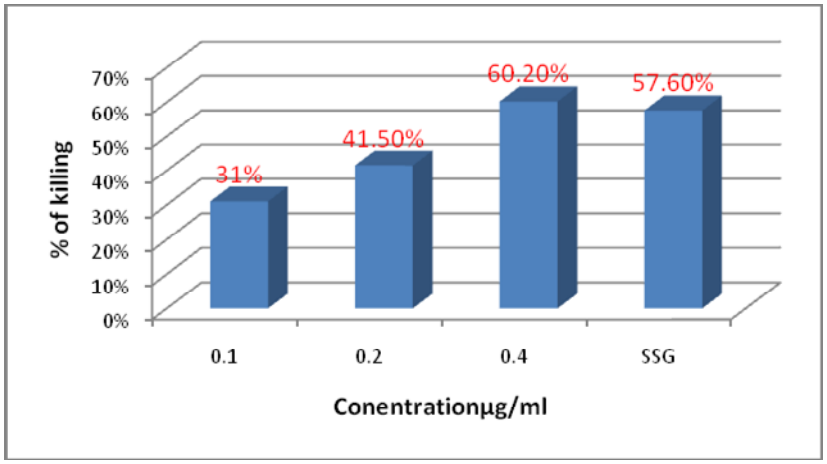

Fig (19): Percentage of killing of *Leishmania major* amastigotes in the presence of isoniazid using in vitro THP1 infection assay.

3.2.3.2.3 Antileishmanial activity of isoniazid plus SSG against *L.donovani* using in vitro macrophage infection assay

To evaluate the antileishmanial activity of INH plus SSG against *leishmania donovani* amastigotes, the following concentrations of INH were added to macrophage infected cells, 0.1, 0.2 and 0.4µg/ml each plus 24µg/ml SSG. The results showed a decrease in the mean number of amastigotes per 100 THP1 and increase in the percentage of killing compared to INH alone treated cells as shown in figure (20 and 21).

Table (10): Mean number of *leishmania donovani* amastigotes per 100 THP1 and percentage of killing produced by 0.1, 0.2 and 0.4 µg/ml of isoniazid alone and 0.1, 0.2 and 0.4µg/ml each plus 24µg/ml SSG and 24µg/ml SSG alone.

Concentration	**Mean No of amastigotes Per 100THP1± SEM**	**% of killing**
0.1	213*±6.1	50.7%
0.2	219*±7.0	53.3%
0.4	201*±5.1	57.1%
0.1+ 24 SSG	184*±3.6	60.8%
0.2+ 24 SSG	143*±3.5	69.5%
0.4+ 24 SSG	130*±5.6	72.3%
SSG	171*±5.1	63.5%
–Ve	469±17.1	

Note: * indicates significance level less than 0.05 ($P < 0.05$).

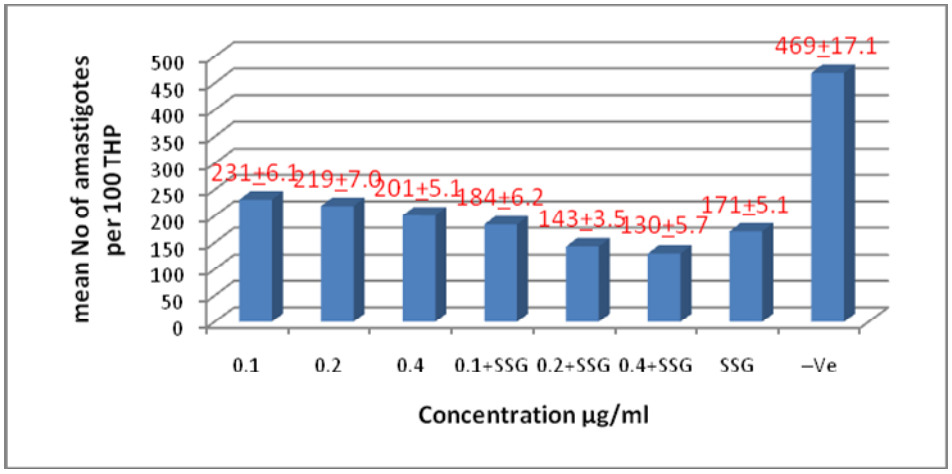

Fig (20): Mean number of *Leishmania donovani* amastigotes per 100 THP1 in the presence of isoniazid plus SSG using in vitro THP1 infection assay.

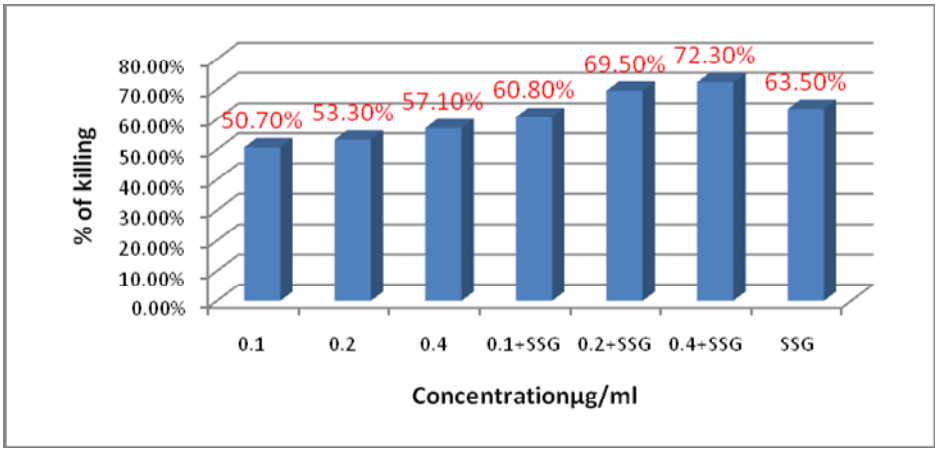

Fig (21): Percentage of killing of *Leishmania donovani* amastigotes in the presence of isoniazid plus SSG using in vitro THP1 infection assay.

3.2.3.2.4 Antileishmanial activity of isoniazid plus SSG against *L.major* using in vitro macrophage infection assay

To evaluate the antileishmanial activity of INH plus SSG against *leishmania major* amastigotes, the following concentrations of INH were added to macrophage infected cells, 0.1, 0.2 and 0.4µg/ml each plus 20µg/ml SSG. The results showed a decrease in the mean number of amastigotes per 100 THP1 and increase in the percentage of killing compared to INH alone treated cells as shown in figure (22 and 23).

Table (11): Mean number of *leishmania major* amastigotes per 100 THP1 and percentage of killing produced by 0.1, 0.2 and 0.4 µg/ml of isoniazid alone and 0.1, 0.2 and 0.4µg/ml each plus 20µg/ml SSG and 20 µg/ml SSG alone.

Concentration	**Mean No of amastigotes Per 100THP1± SEM**	**% of killing**
0.1	260*±6.3	34.7%
0.2	220*±10.1	42.4%
0.4	153*±8.1	61.2%
0.1+ 20 SSG	107*±9.5	72.8%
0.2+ 20 SSG	88*±9.2	77.7%
0.4+ 20 SSG	64*±6.5	83.8%
20 SSG	128*±9.2	67.5%
–Ve	394±8.7	

Note: * indicates significance level less than 0.05 ($P < 0.05$).

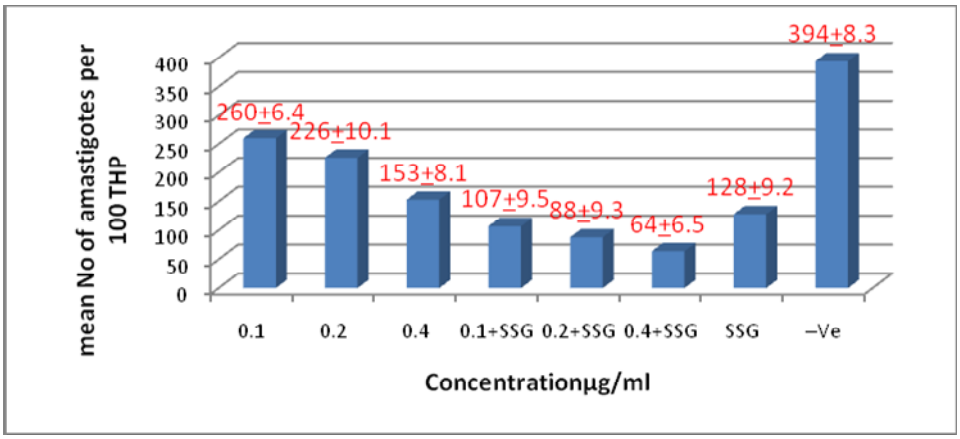

Fig (22): Mean number of *Leishmania major* amastigotes per 100 THP1 in the presence of isoniazid plus SSG using in vitro THP1 infection assay.

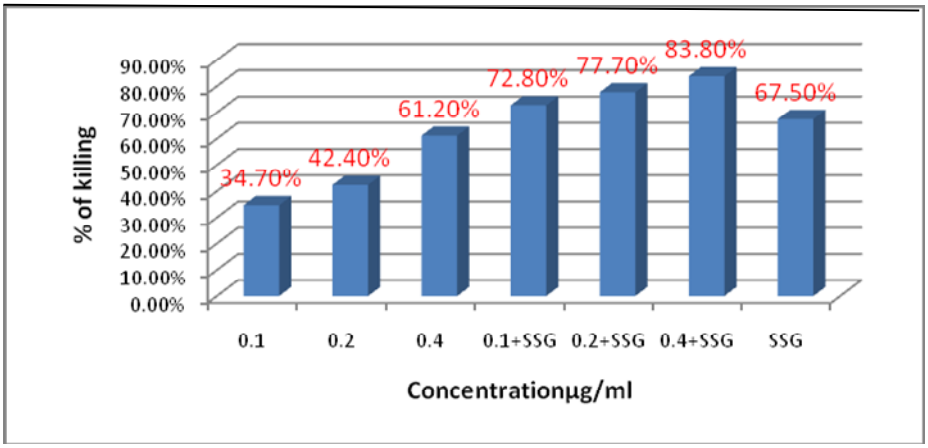

Fig (23): Percentage of killing of *Leishmania major* amastigotes in the presence of isoniazid plus SSG using in vitro THP1 infection assay.

3.2.3.2.5 Antileishmanial activity of isoniazid plus amBisome against *L.donovani* using in vitro macrophage infection assay

To evaluate the antileishmanial activity of INH plus amBisome against *leishmania donovani* amastigotes, the following concentrations of INH were added to macrophage infected cells, 0.1, 0.2 and 0.4µg/ml each plus 10µg/ml amBisome. The results showed a decrease in the mean number of amastigotes per 100 THP1 and increase in the percentage of killing compared to INH alone treated cells as shown in figure (24 and 25).

Table (12): Mean number of *leishmania donovani* amastigotes per 100 THP1 and percentage of killing produced by 0.1, 0.2 and 0.4µg/ml of isoniazid alone and 0.1, 0.2 and 0.4µg/ml each plus 10µg/ml amBisome and 10µg/ml amBisome alone.

Concentration	**Mean No of amastigotes Per 100THP1± SEM**	**% of killing**
0.1	193* ± 4.0	48%
0.2	178* ±5.2	52%
0.4	166* ±3.8	55.3%
0.1+10amb	119* ±5.5	67.9%
0.2+ 10amb	110 *±8.1	70.4%
0.4+10amb	92* ±4.7	75.2%
10 amb	153* ±4.2	58.8%
–Ve	371 ±6.2	

Note: * indicates significance level less than 0.05 ($P < 0.05$).

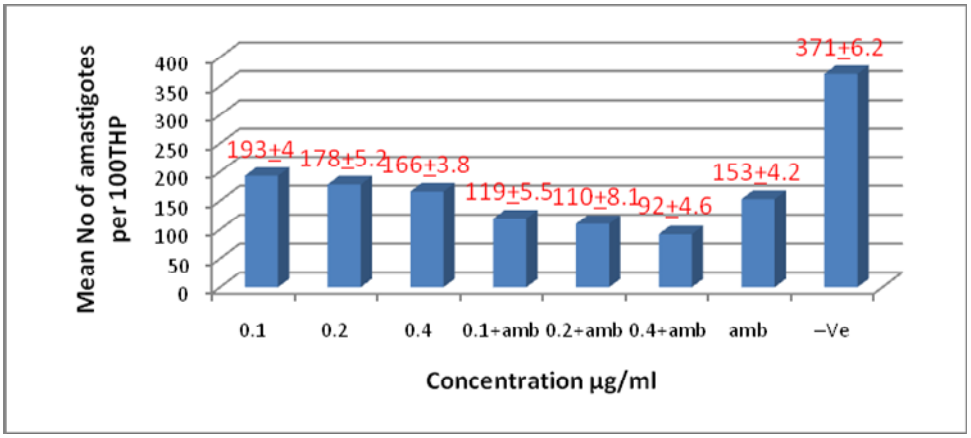

Fig (24): Mean number of *Leishmania donovani* amastigotes per 100 THP1 in the presence of isoniazid plus amBisome using in vitro THP1 infection assay.

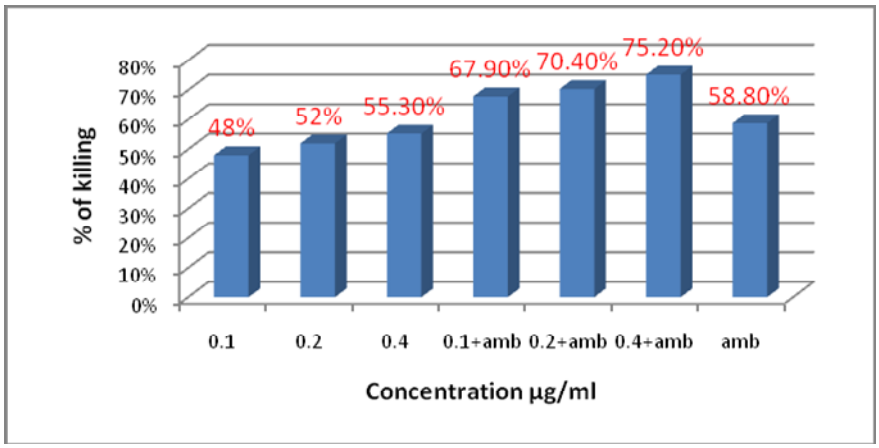

Fig (25): Percentage of killing of *Leishmania donovani* amastigotes in the presence of isoniazid plus amBisome using in vitro THP1 infection assay.

3.2.3.2.6 Antileishmanial activity of isoniazid plus amBisome against *L.major* using in vitro macrophage infection assay

To evaluate the antileishmanial activity of INH plus amBisome against *leishmania major* amastigotes, the following concentrations of INH were added to macrophage infected cells, 0.1, 0.2 and 0.4µg/ml each plus 10µg/ml amBisome. The results showed a decrease in the mean number of amastigotes per 100 THP1 and increase in the percentage of killing compared to INH alone treated cells as shown in figure (26 and 27).

Table (13): Mean number of *leishmania major* amastigotes per 100 THP1 and percentage of killing produced by 0.1, 0.2 and 0.4µg/ml of isoniazid alone and 0.1, 0.2 and 0.4µg/ml each plus 10µg/ml amBisome and 10µg/ml amBisome alone.

Concentration	**Mean No of amastigotes Per 100THP1± SEM**	**% of killing**
0.1	228*±8.2	30.3%
0.2	201*±11.8	38.5%
0.4	17*9±9.2	45.3%
0.1+10amb	115*±8.1	64.8%
0.2+ 10amb	96*±9.3	70.6%
0.4+10amb	75*±11.2	77.1%
10 amb	162*±6.2	50.5%
–Ve	327±11.1	

Note: * indicates significance level less than 0.05 ($P < 0.05$).

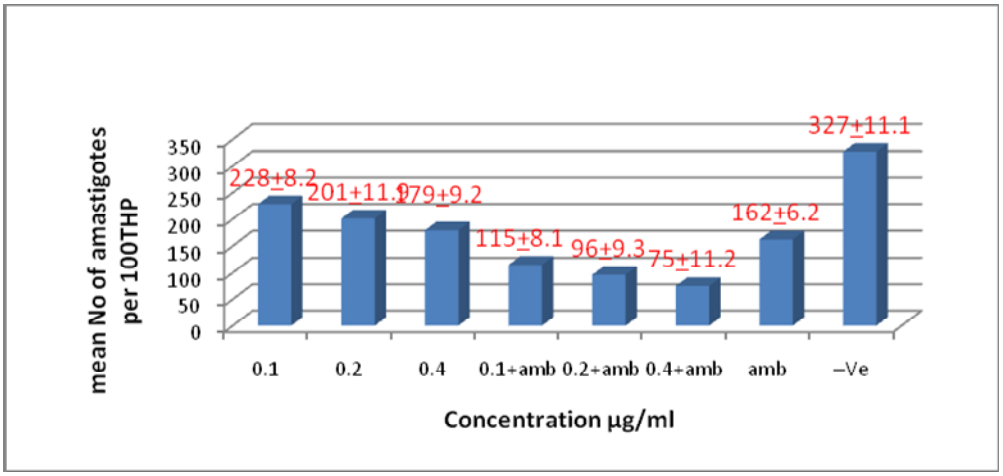

Fig (26): Mean number of *Leishmania major* amastigotes per 100 THP1 in the presence of isoniazid plus amBisome using in vitro THP1 infection assay.

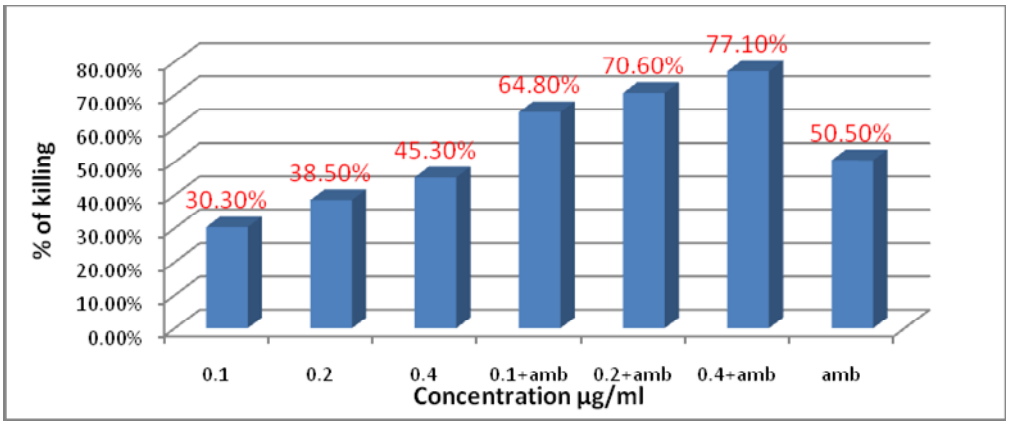

Fig (27): Percentage of killing of *Leishmania major* amastigotes in the presence of isoniazid plus amBisome using in vitro THP1 infection assay.

3.2.3.2.7 Antileishmanial activity of Sulphadoxine plus pyrimethamine against *L.donovani* using in vitro macrophage infection assay

To evaluate the antileishmanial activity of Sulphadoxine plus pyrimethamine against *leishmania donovani* amastigotes, the following concentrations were added to macrophage infected cells, 1.9 pyrimethamine and 38.1 sulfadoxine, 2.9 pyrimethamine and 57.1 sulfadoxine and 3.8 pyrimethamine and 76.2sulfadoxine The results showed a decrease in the mean number of amastigotes per 100 THP1 as compared to the untreated cells (negative control) and increase in the percentage of killing as shown in figure (28 and 29).

Table (14): Mean number of *leishmania donovani* amastigotes per 100 THP1 and percentage of killing produced by different concentrations of Sulphadoxine plus pyrimethamine and 24µg/ml of SSG.

Concentration	**Mean No of amastigotes Per 100THP1± SEM**	**% of killing**
1	373*±7.2	27.6%
2	350*±5.7	32%
3	317*±7.8	38.5%
24 SSG	212*±10.4	58.8%
–Ve	515±14.5	

Note: * indicates significance level less than 0.05 ($P < 0.05$).

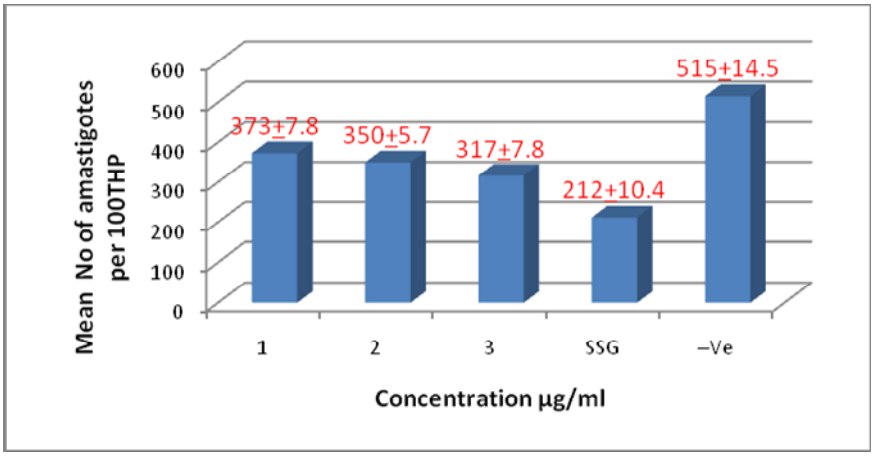

Fig (28): Mean number of *Leishmania donovani* amastigotes per 100 THP1 in the presence of sulfadoxine plus pyrimethamine using in vitro THP1 infection assay.

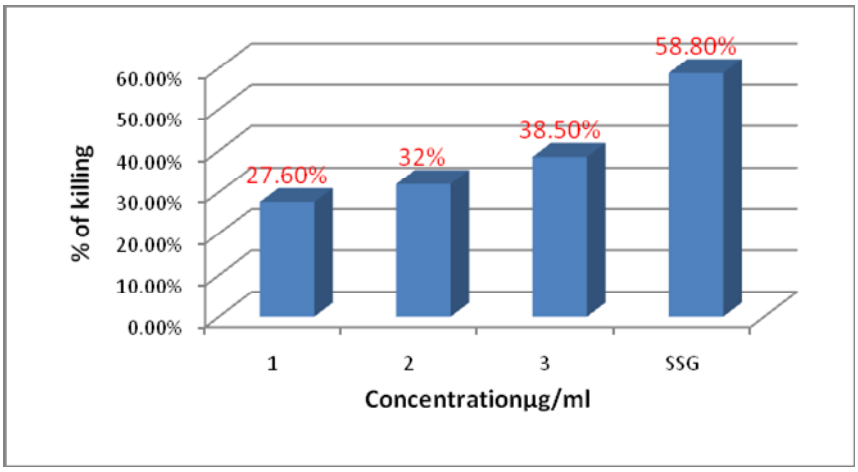

Fig (29): Percentage of killing of *Leishmania donovani* amastigotes in the presence of sulfadoxine plus pyrimethamine using in vitro THP1 infection assay.

3.2.3.2.8 Antileishmanial activity of Sulphadoxine plus pyrimethamine against *L.major* using inviro macrophage infection

To evaluate the antileishmanial activity of Sulphadoxine plus pyrimethamine against *leishmania majori* amastigotes, the following concentrations were added to macrophage infected cells, 1.9 pyrimethamine and 38.1 sulfadoxine, 2.9 pyrimethamine and 57.1 sulfadoxine and 3.8 pyrimethamine and 76.2sulfadoxine The results showed a decrease in the mean number of amastigotes per 100 THP1 as compared to the untreated cells (negative control) and increase in the percentage of killing as shown in figure (30 and 31).

Table (15): Mean number of *leishmania major* amastigotes per 100 THP1 and percentage of killing produced by different concentrations of Sulphadoxine plus pyrimethamine and 24µg/ml of SSG.

Concentration	**Mean No of amastigotes Per 100THP1± SEM**	**% of killing**
1	342*±4.7	28.9%
2	308*±5.9	35.9%
3	286*±7.0	40.9%
20 SSG	189*±6.0	60.7%
–Ve	481±9.2	

Note: * indicates significance level less than 0.05 ($P < 0.05$).

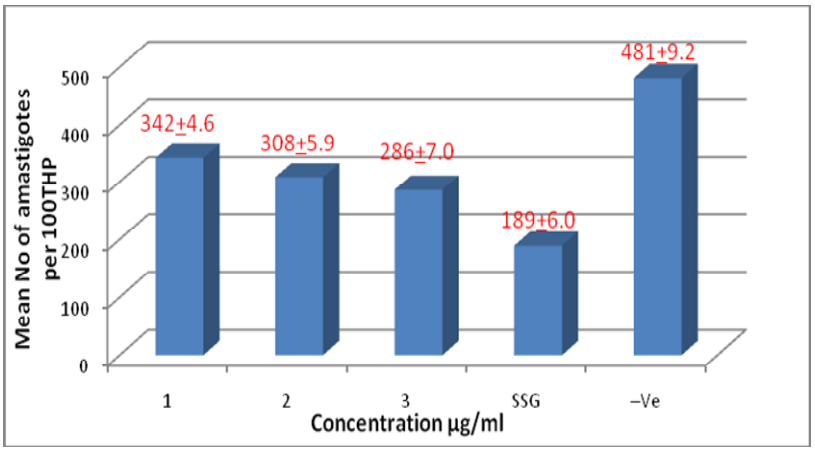

Fig (30): Mean number of *Leishmania major* amastigotes per 100 THP1 in the presence of sulfadoxine plus pyrimethamine using in vitro THP1 infection assay.

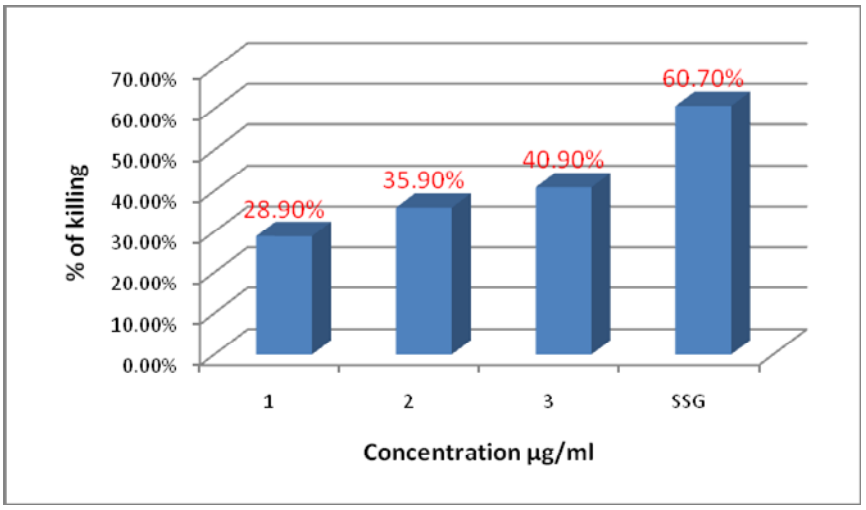

Fig (31): Percentage of killing of *Leishmania major* amastigotes in the presence of sulfadoxine plus pyrimethamine using in vitro THP infection assay.

Statistics

Paired Sample Test

L.donovani					L.major				
Negative Vs	**S.D**	**S.E**	**Tvalue**	**sig**	**Negative Vs**	**S.D**	**S.E**	**Tvalue**	**sig**
0.1 INH	**22.1**	**12.8**	**-32.7**	**.001**	**0.1 INH**	**39.9**	**23.1**	**-10.5**	**.009**
0.2 INH	**30.1**	**17.3**	**-24.9**	**.002**	**0.2 INH**	**62.3**	**36.0**	**-9.1**	**.012**
0.4 INH	**17.6**	**10.1**	**-45.3**	**.000**	**0.4 INH**	**77.7**	**44.9**	**-9.8**	**.010**
0.1 INH + SSG	**26.8**	**15.5**	**-18.4**	**.003**	**0.1 INH + SSG**	**8.5**	**4.9**	**-58.2**	**.000**
0.2 INH + SSG	**35.3**	**20.4**	**-16.0**	**.004**	**0.2 INH + SSG**	**30.0**	**17.3**	**-16.7**	**.004**
0.4 INH + SSG	**26.1**	**15.0**	**-22.5**	**.002**	**0.4 INH + SSG**	**4.0**	**2.3**	**-142.9**	**.000**
0.1 INH + amB	**31.0**	**17.9**	**-11.8**	**.007**	**0.1 INH + amB**	**20.2**	**11.7**	**-21.6**	**.002**
0.2 INH + amB	**16.5**	**9.5**	**-24.3**	**.002**	**0.2 INH + amB**	**24.2**	**14.0**	**-18.6**	**.003**
0.4 INH + amB	**37.4**	**21.6**	**-11.7**	**.007**	**0.4 INH + amB**	**3.6**	**2.1**	**-134.0**	**.000**

L.donovani					L.major				
SSG Vs	S.D	S.E	Tvalue	sig	SSG Vs	S.D	S.E	Tvalue	sig
0.1 INH	34.0	19.6	4.9	.039	0.1 INH	64.2	37.1	11.9	.007
0.2 INH	26.2	15.1	5.6	.030	0.2 INH	87.1	50.2	7.8	.016
0.4 INH	38.6	22.3	2.4	.133	0.4 INH	52.4	30.2	-4.7	.042
0.1 INH + SSG	3.6	2.1	1.7	.228	0.1 INH + SSG	31.2	18.0	-1.2	.364
0.2 INH + SSG	19.5	11.2	-13.5	.005	0.2 INH + SSG	9.5	5.5	-4.2	.050
0.4 INH + SSG	17.4	10.1	-4.4	.048	0.4 INH + SSG	27.2	15.7	-4.1	.053

L.donovani					L.major				
amBisome Vs	S.D	S.E	Tvalue	sig	amBisom Vs	S.D	S.E	Tvalue	sig
0.1 INH + amB	3.6	2.1	-22.5	.002	0.1 INH + amB	6.2	3.6	-9.4	.011
0.2 INH + amB	19.4	11.2	-5.9	.028	0.2 INH + amB	11.1	6.4	-6.7	.022
0.4 INH + amB	17.4	10.1	-8.6	.013	0.4 INH + amB	15.1	8.7	-7.0	.020

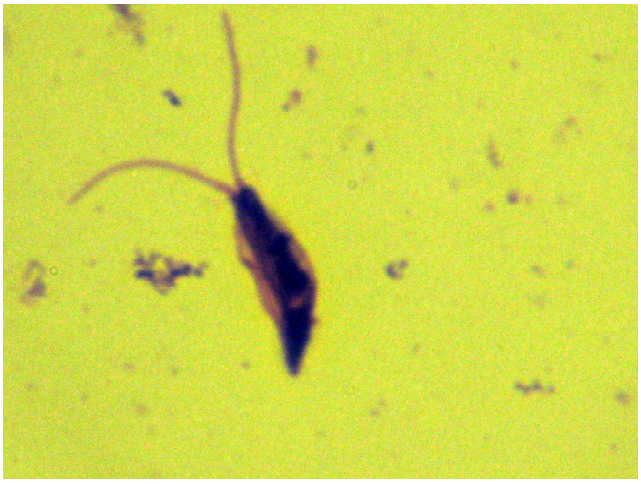

Fig (32): Culture of *L.major* promastigotes in DMEM complete media, Geimsa stain X100

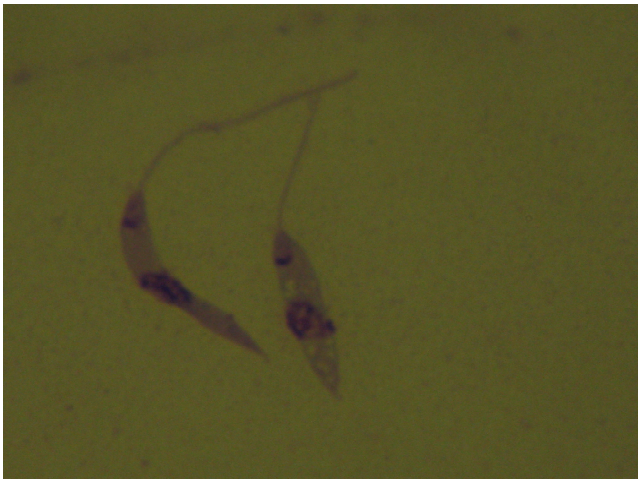

Fig (33): Culture of *L.donovani* promastigotes in DMEM complete media, Geimsa stain X100

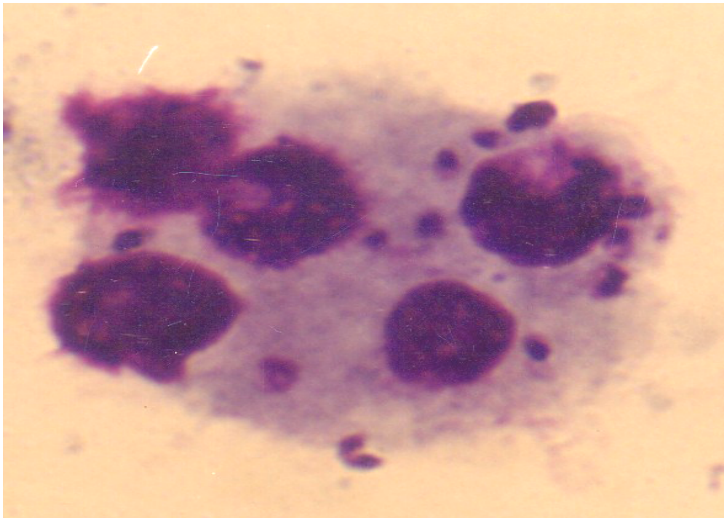

Fig (34): Infection of macrophages human cell line THP1 with *L.major* promastigotes, 3days post infection. Geimsa stainX100

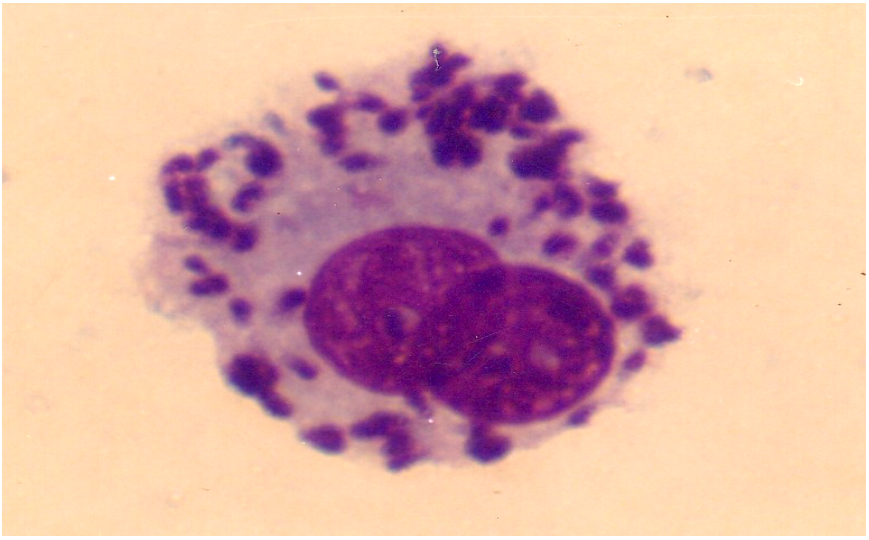

Fig (35): Infection of macrophages human cell line THP1 with *Ldonovani* promastigotes, 3post infection. Geimsa stainX100

Chapter four

Disscussion

Leishmaniasis is a major public health problem in Sudan and different clinical forms are endemic in many areas of the country (Zijlstra and El Hassan, 2001). It is difficult to treat and there is increasing resistance developing against the currently available drugs (Davidson, 1999). Sodium stibogluconate (SSG) is the first line of treatment of visceral leishmaniasis in Sudan. Liposomal amphotericin B is the second line treatment. Currently combination of sodium stibogluconate and liposomal amphotericin B is under trial.

In this study the tested Leishmania isolates were cultured from the parasite cryo bank in the institute of endemic diseases and previously typed by multienzyme electrophoresis. The two isolates were characterized in this study by KDNA PCR amplification. The result showed an amplicon of 800 bp of L. donovani complex and an amplicon of 700 bp of L. major complex as previously reported by Smyth et al (1992). The in vitro culture of the promastigotes of the two species was achieved in both the triple N and DMEM media.

In vitro infection of human macrophage THP1 is a widely used method for in vitro evaluation of new anti leishmanial drugs and for testing the drug sensitivity of the parasite. A significant parasite infection by L. donovani and L. major promastigotes was achieved in this study.

The completion of the sequencing of the whole genome and the analysis of the proteomes of leishmania, mycobacteria and plasmodium opens access to valuable data that can be used for diagnostics, drugs and vaccine discovery. The aim of this

study was to identify new drug targets for production of safe, effective and affordable drugs using comparative bioinformatics and to determine the sensitivity of the selected drugs against leishmania using in vitro drug sensitivity assay. Leishmaina and mycobacteria belong to different phyla, the former is a protozoan of the family trypansomatidae and the latter is a bacteria belonging to Mycobacteriacae, while plasmodium belongs to the family plasmodiidae.

Interestingly alignment of mycobacterium fatty acid synthase and leishmania major and leishmania donovani 3-oxoacyl- acyl carrier protein synthaseII showed 50% maximum identities. The mycobacterium fatty acid synthase enzyme that is responsible for formation of mycolic acid which constitutes the highly impermeable outer lipid layer of the cell wall (Dubnau *et al*., 2000) is inhibited by isoniazid (Takayma *et al.,* 1972). Leishmania 3-oxoacyl- acyl carrier protein synthase II enzyme is involved in fatty acid synthesis too. The identified homology suggested possible effect of isoniazid; a drug used in combination with rifampicin for treatment of pulmonary tuberculosis; against Leishmanaia parasite. Isoniazid showed significant effect ($p<0.05$) against *Leishmania major* amastigotes at concentrations of 0.1, 0.2 and 0.4µg/ml compared with 20µg/ml SSG and with the negative controls using in vitro THP infection assay. Doses of 0.1 and 0.2µg/ml isoniazid produced lesser percentage of killings than that produced by 20µg/ml SSG, while dose of 0.4µg/ml produced greater percentage of killing (60.2%) than that produced by 20µg/ml SSG (57.6%).

Isoniazid also showed significant effect ($p<0.05$) against *Leishmania donovani* amastigotes at concentrations of 0.1, 0.2 and 0.4µg/ml compared with 24µg/ml SSG and with the negative controls using in vitro THP infection assay. But the three concentrations produced lesser percentage of killing (52.8, 54.3 and 58.8%) than that produced by 24µg/ml SSG and greater than that on *Leishmania major* produced by the same dose suggesting isoniazid to be more active against *Leishmania donovani* amastigotes.

A previous report (Berman and Lee, 1983) showed an antileshmainial effect of isoniazid. It was tested in combination with rifampicin against Leishmania tropica within human macrophages in vitro and it produced 40% parasite elimination.

A synergistic effect of INH and SSG against *Leishmania major* amastigotes was evident by the increase in the percentage of killing from 34.7, 42.4 and 61.2% to 72.8, 77.7 and 83.8% when the two drugs were added together to the test cultures. This synergistic effect was evident by little increase in the percentage of killing of *Leishmania donovani* amastigotes from 50.7, 53.3 and 57.1% to 60.8, 69.5 and 72.3% encouraging the use of SSG plus isoniazid for invivo treatment of leishmaniasis.

Another synergism was obtained when INH and amBisome were added together. This was evident by the increase in the percentage of killing of *Leishmania major* amastigotes from 30.3, 38.5 and 45.3% to 64.8, 70.6 and 77.1% and the

percentage of killing of *Leishmania donovani* amastigotes from 48.0, 52.0 and 55.3% to 67.9, 70.4 and 75.2% when the two drugs were added together to the test cultures suggesting administration of INH together with amBisome could eliminate more parasite than amBisome alone.

Addition of SSG to isoniazid produced more increase in the percentage of killing of both amastigotes of *Leishmania major* and *Leishmania donovani* as compared to that produced by addition of amBisome to isoniazid.

Alignment of dihydrofolate reductase-thymidylate synthase of plasmodium falciparum and Leishmania donovani showed 53% maximum identities, while with that of plasmodium vivax showed 52% maximum identities. Alignment of dihydrofolate reductase-thymidylate synthase of plasmodium falciparum and Leishmania major showed 53% maximum identities, while with that of plasmodium vivax showed only 27% maximum identities. This bifunctional enzyme catalyses the reduction of folate to dihydrofolat and subsequently to tetrahydrofolate (Beverley, 1991: Webber and Whiteley, 1985).

Pyrimethamine plus sulfadoxine produces marked activity reduction in the promastigotes. But it was less active against leishmania amastigotes (less than 41% percentage of killing in both *Leishmania major* and *Leishmania donovani* amastigotes) compared to SSG. Previous report (De Carvalho *et al.*, 2003) showed antileshmainial effect of targeted drugs of pyrimethamine such as carboxymethyldextranthio-mannopyranoside pyrimethamine. It was tested at dose

of 4.86µg/ml pyrimethamine against *Leishmania amzonensis* amastigotes within human macrophages in vitro and it produced 50% destruction of intracellular amastigotes.

Pyrimethamine sulfadoxine combination is capable of inhibition of dihydrofolate rteductase thymidylate synthase (DHFR? TS) which catalyses the production of tetrahydrofolate and dTMP which are crucial for pyrimidine biosynthesis. The less activity of this antifolate may be due to pteridine reductase 1(PTR1), a novel leishmania enzyme responsible for salvage of pteridine, it catalyses the reduction of conjugated and unconjugated pterins and it can carry out the reduction of folate to dihydro and tetrahydrofolate (Nare *et al.,* 1997). Hence PTR1 is much less sensitive to sulfadoxine than DHFR–TS (Bello *et al.*, 1994); it can provide a metabolic by pass to the blocked DHFR–TS and mediates antifolate resistance although it is inhibited by pyrimethamine but this effect is produced in high concentration.

The effect of isoniazid alone and in combination with SSG or amBisome is very promising and this should be further evaluated. Isoniazid plus SSG or amBisome could lead to reduction in the treatment course hence isoniazid is a safe drug and already in use for the treatment of pulmonary tuberculosis. Isoniazid is safe, affordable and administered orally and could be used in Combination to enhance the cure and prevent the emergence of drug resistance.

Conclusion

- Isoniazid, the anti tuberculus drug, has antileishmanial activity at 0.1, 0.2 and 0.4µg/ml concentrations in vitro.
- The antileishmanial activity of sodium stibogluconate is synergistically increased by isoniazid.
- The antileishmanial activity of liposomal amphotericin B is synergistically increased by isoniazid.
- Sulfadoxine pyrimethamine combination has minimum antileishmanial activity at 1.9 pyrimethamine and 38.1 sulfadoxine, 2.9 pyrimethamine and 57.1sulfadoxine, 3.8 pyrimethamine and 76.2sulfadoxine concentrations in vitro.

Recommendations

- Evaluation of the antileishmanial activity of isoniazid alone and in combination with sodium stibogluconate or amBisome in vivo.
- Evaluation of the antileishmanial activity of Sulfadoxine pyrimethamine combination using higher concentration in vitro.
- Evaluation of the antileishmanial activity of Sulfadoxine pyrimethamine combination in vivo.

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Appendix 1

Alignment of leishmania major 3-oxoacyl- acyl carrier protein synthase¡¡ and mycobacterium fatty acid synthase

```
Score = 55.5 bits (132), Expect = 3e-11, Method: Compositional
matrix adjust.
 Identities = 87/322 (27%), Positives = 130/322 (40%), Gaps =
43/322 (13%)
Query  157   GQVYYSKVPPMFLPKILGNMAAGNTAIRHKLRGPIGSS------
VAACATGAHCIGEAAS  210
             G +     P     ++L N+ A      H ++  +GS       VAACAT A
+ E
Sbjct  2678  GNLLGRNKPNDIFQEVLPNIIAA-----
HVVQSYVGSYGAMIHPVAACATAAVSVEEGVD  2732
Query  211   WIREGRADVMVCGAAEACITPVSVAGFTRMRA------
LCTRYNETPSSASRPLDITRAG  264
              IR G+A ++V G  +  +T   + GF  M A      +C R     S
SRP D  R G
Sbjct  2733  KIRLGKAQLVVAGGLD-DLTLEGIIGFGDMAATADTSMMCGR-
GIHDSKFSRPNDRRRLG  2790
Query  265   FVMGEGAGVLILEALEHAVARAAP--
RVYAELRGFGISCDAHHVAVPHPDGLGARRC---  319
             FV  +G G ++L   + A+    P   V A  + FG   D  H ++P P
Sbjct  2791  FVEAQGGGTILLARGDLALRMGLPVLAVVAFAQSFG---
DGVHTSIPAPGLGALGAGRGG  2847
Query  320   ----
VEQALADGGDVPATAVGYVNAHATGTIGDEVELMAIQRALRPSTAPSSTA-LHVSS  374
                + +ALA  G V A  V  ++ H T T+ ++     +   L  +   S
A L V S
Sbjct  2848  KDSPLARALAKLG-
VAADDVAVISKHDTSTLANDPNETELHERLADALGRSEGAPLFVVS  2906

Query  375   AKGGLGHLLGAAGSVEAALTVLALHEQRAPPTANLTTSCLTKEQQDCG-
LVCIQGS----  429
              K   GH  G A   +       L +   PP  N +  C+  E       V
++ +
Sbjct  2907  QKSLTGHAKGGAAVFQMMGLCQILRDGVIPP--
NRSLDCVDDELAGSAHFVWVRDTLRLG  2964

Query  430   AAQPLQSCEAAISTSFGFGGIN  451
```

```
               PL+   A + TS GFG ++
Sbjct  2965  GKFPLK---AGMLTSLGFGHVS  2983

 Score = 23.1 bits (48),  Expect = 0.13, Method: Compositional
matrix adjust.
 Identities = 21/72 (29%), Positives = 32/72 (44%), Gaps = 2/72
(3%)
Query  350   VELMAIQRALRPSTAPSSTALHVSSAKGGLGHLLGAAGSVEAALTV--
LALHEQRAPPTA  407
             +EL+A Q A       +   L +  A GGLG        V+++ TV  LA +
+ P  A
Sbjct  1650
IELLAWQFASPVRWIETQDLLFIEEAAGGLGVERFVEIGVKSSPTVAGLATNTLKLPEYA
1709
Query  408   NLTTSCLTKEQQ  419
             + T   L  E+
Sbjct  1710  HSTVEVLNAERD  1721

 Score = 20.4 bits (41),  Expect = 0.97, Method: Compositional
matrix adjust.
 Identities = 10/15 (67%), Positives = 10/15 (67%), Gaps = 2/15
(13%)

Query  228  CITP--VSVAGFTRM  240
            CI P   SVAG TRM
Sbjct  876  CIIPGTASVAGITRM  890

 Score = 18.1 bits (35),  Expect = 4.6, Method: Compositional
matrix adjust.
 Identities = 11/34 (32%), Positives = 20/34 (59%), Gaps = 1/34
(3%)
Query  379  LGHLLGAAGSVEAALTVLALHEQRAPPTANLTTS  412
            L  L+GAAG++ A    +++   R PP  ++T +
Sbjct  171  LAQLIGAAGTLVARRRGISVLGDR-PPMVSVTNA  203

 Score = 17.3 bits (33),  Expect = 8.3, Method: Compositional
matrix adjust.
 Identities = 9/18 (50%), Positives = 11/18 (61%), Gaps = 0/18
(0%)

Query  268  GEGAGVLILEALEHAVAR  285
            G   GVL +EAL+   AR
Sbjct  147  GHSQGVLAVEALKAGGAR  164
```

Alignment of leishmania donovani 3-oxoacyl- acyl carrier protein synthase¡¡ and mycobacterium fatty acid synthase

```
Score = 54.7 bits (130), Expect = 5e-11, Method: Compositional
matrix adjust.
 Identities = 79/284 (28%), Positives = 119/284 (42%), Gaps =
37/284 (13%)
Query  157   GHVHYSKVPPMFLPKILGNMAAGNTAIRHKLRGPIGSS------
VAACATGAHCIGEAAL  210
             G++     P     ++L N+ A      H ++  +GS       VAACAT A
+ E
Sbjct  2678  GNLLGRNKPNDIFQEVLPNIIAA-----
HVVQSYVGSYGAMIHPVAACATAAVSVEEGVD  2732
Query  211   WIREGRADVMVCGAAEACITPVSVAGFTRMRA------LCTR-
FNDTPSSASRPFDITRA  263
              IR G+A ++V G  +  +T   + GF  M A      +C R  +D  S
SRP D  R
Sbjct  2733  KIRLGKAQLVVAGGLD-DLTLEGIIGFGDMAATADTSMMCGRGIHD--
SKFSRPNDRRRL  2789
Query  264   GFVMGEGAGVLILEALEHAVARAAP--
RVYAELRGFGISCDAHHVAVPHPEGLGARRC--  319
             GFV  +G G ++L   + A+    P   V A  + FG   D  H ++P P
Sbjct  2790  GFVEAQGGGTILLARGDLALRMGLPVLAVVAFAQSFG---
DGVHTSIPAPGLGALGAGRG  2846
Query  320   -----
VEQALAEGGDVPASAVGYVNAHATGTIGDEVELMAIQQALRPSTAPSSPA-LHVS  373
                  + +ALA+ G V A  V  ++ H T T+ ++     + + L  +   S
A L V
Sbjct  2847  GKDSPLARALAKLG-
VAADDVAVISKHDTSTLANDPNETELHERLADALGRSEGAPLFVV  2905

Query  374   SAKGGLGHLLGAAGSVEAALTVLALHEQRAPPTANLTTSCLTRE  417
             S K   GH  G A   +       L +   PP  N +  C+  E
Sbjct  2906  SQKSLTGHAKGGAAVFQMMGLCQILRDGVIPP--NRSLDCVDDE  2947

 Score = 22.3 bits (46),  Expect = 0.23, Method: Compositional
matrix adjust.
 Identities = 21/72 (29%), Positives = 32/72 (44%), Gaps = 2/72
(3%)
Query  350   VELMAIQQALRPSTAPSSPALHVSSAKGGLGHLLGAAGSVEAALTV--
LALHEQRAPPTA  407
             +EL+A Q A       +   L +  A GGLG        V+++ TV  LA +
+ P  A
```

```
Sbjct  1650
IELLAWQFASPVRWIETQDLLFIEEAAGGLGVERFVEIGVKSSPTVAGLATNTLKLPEYA
1709
Query  408   NLTTSCLTREQQ  419
             + T   L  E+
Sbjct  1710  HSTVEVLNAERD  1721
```

Score = 20.4 bits (41), Expect = 0.81, Method: Compositional matrix adjust.
Identities = 13/27 (48%), Positives = 13/27 (48%), Gaps = 6/27 (22%)

```
Query  228  CITP--VSVAGFTRMR----ALCTRFN  248
            CI P   SVAG TRM      L  RF
Sbjct  876  CIIPGTASVAGITRMDEPVGELLDRFE  902
```

Score = 18.1 bits (35), Expect = 4.1, Method: Compositional matrix adjust.
Identities = 11/34 (32%), Positives = 20/34 (59%), Gaps = 1/34 (3%)

```
Query  379  LGHLLGAAGSVEAALTVLALHEQRAPPTANLTTS  412
            L  L+GAAG++ A    +++   R PP  ++T +
Sbjct  171  LAQLIGAAGTLVARRRGISVLGDR-PPMVSVTNA  203
```

Score = 17.3 bits (33), Expect = 8.1, Method: Compositional matrix adjust.
Identities = 9/18 (50%), Positives = 11/18 (61%), Gaps = 0/18 (0%)

```
Query  268  GEGAGVLILEALEHAVAR  285
            G   GVL +EAL+   AR
Sbjct  147  GHSQGVLAVEALKAGGAR  164
```

Alignment of dihydrofolate reductase-thymidylate synthase [Leishmania major] and dihydrofolate reductase-thymidylate synthase [Plasmodium falciparum]

```
Score = 341 bits (875), Expect = 4e-98, Method: Compositional
matrix adjust.
 Identities = 153/288 (53%), Positives = 205/288 (71%), Gaps =
3/288 (1%)

Query  232
HEERQYLELIDRIMKTGIVKEDRTGVGTISLFGAQMRFSLRDNRLPLLTTKRVFWRGVCE  291
            H E QYL +I  IM  G  + DRTGVG +S FG  M+F L
PLLTTK++F RG+ E
Sbjct  323  HPEYQYLNIIYDIMMNGNKQSDRTGVGVLSKFGYIMKFDLSQ-
YFPLLTTKKLFLRGIIE  381

Query  292
ELLWFLRGETSAQLLADKDIHIWDGNGSREFLDSRGLTENKEMDLGPVYGFQWRHFGADY  351
            ELLWF+RGET+   L +K++ IW+ NG+REFLD+R L   +
DLGP+YGFQWRHFGA+Y
Sbjct  382
ELLWFIRGETNGNTLLNKNVRIWEANGTREFLDNRKLFHREVNDLGPIYGFQWRHFGAEY  441

Query  352
KGFEANYDGEGVDQIKLIVETIKTNPNDRRLLVTAWNPCALQKMALPPCHLLAQFYVNTD  411
                NY+ +GVDQ+K I+  IK +P  RR+L+ AWN   L +MALPPCH+L
QFYV
Sbjct  442
TNMYDNYENKGVDQLKNIINLIKNDPTSRRILLCAWNVKDLDQMALPPCHILCQFYV--F  499

Query  412
TSELSCMLYQRSCDMGLGVPFNIASYALLTILIAKATGLRPGELVHTLGDAHVYRNHVDA  471
             +LSC++YQRSCD+GLGVPFNIASY++ T +IA+   L+P + +H
LG+AHVY NH+D+
Sbjct  500
DGKLSCIMYQRSCDLGLGVPFNIASYSIFTHMIAQVCNLQPAQFIHVLGNAHVYNNHIDS  559

Query  472  LKAQLERVPHAFPTLIFKEERQYLEDYELTDMEVIDYVPHPAIKMEMA  519
            LK QL R+P+ FPTL    + + +ED+ ++D  + +YV H  I M+MA
Sbjct  560  LKIQLNRIPYPFPTLKLNPDIKNIEDFTISDFTIQNYVHHEKISMDMA  607

 Score = 76.6 bits (187), Expect = 2e-18, Method: Compositional
matrix adjust.
```

```
 Identities = 60/213 (28%), Positives = 93/213 (44%), Gaps =
48/213 (23%)
Query  38   GIGDGESIPWRVPE-DMTFFKNQTTLLR----------------------
NKKPPTEKK  73
            G+G+   +PW+    DM +F   TT +                        N
P ++K
Sbjct  39
GLGNKGVLPWKCNSLDMKYFCAVTTYVNESKYEKLKYKRCKYLNKETVDNVNDMPNSKKL  98
Query  74
RNAVVMGRKTWESVPVKFRPLKGRLNIVLSSKATVEELLAPLPEGQRAAAAQDVVVVNGG  133
            +N VVMGR  WES+P KF+PL  R+N++LS     E+
+DV ++N
Sbjct  99   QNVVVMGRTNWESIPKKFKPLSNRINVILSRTLKKEDF------------
DEDVYIIN-K  145
Query  134
LAEALRLLARPLYCSSIETAYCVGGAQVYADAMLSPCIEKLQEVYLTRIYATAPACTRFF  193
            + + + LL +  Y       + +GG+ VY + +    I+K   +Y TRI +T
C  FF
Sbjct  146  VEDLIVLLGKLNY----YKCFIIGGSVVYQEFLEKKLIKK---
IYFTRINSTY-ECDVFF  197

Query  194  PFPPENAATAWDLASSQGRRKSEAEGLEFEICK  226
            P   EN    + + S      S    L+F I K
Sbjct  198  PEINEN---EYQIISVSDVYTSNNTTLDFIIYK  227

 Score = 18.9 bits (37),  Expect = 0.58, Method: Compositional
matrix adjust.
 Identities = 7/19 (37%), Positives = 12/19 (63%), Gaps = 2/19
(11%)
Query  400  CHL--LAQFYVNTDTSELS  416
            CH+  L +FY N D  +++
Sbjct  259  CHMKKLTEFYKNVDKYKIN  277

 Score = 16.9 bits (32),  Expect = 2.2, Method: Compositional
matrix adjust.
 Identities = 6/20 (30%), Positives = 11/20 (55%), Gaps = 0/20
(0%)
Query  82   KTWESVPVKFRPLKGRLNIV  101
            + + S+  K+ P    LNI+
Sbjct  313  QIYNSLKYKYHPEYQYLNII  332
```

Alignment of dihydrofolate reductase-thymidylate synthase [Leishmania major] and dihydrofolate reductase-thymidylate synthase [Plasmodium vivax]

```
Score = 65.1 bits (157), Expect = 2e-15, Method: Compositional
matrix adjust.
 Identities = 49/187 (27%), Positives = 81/187 (44%), Gaps =
50/187 (26%)

Query  37   HGIGDGESIPWRVPE-DMTFFKNQTTLLRNKK----------------------
-------  67
             G+G+  ++PW+    DM +F++ TT +   K
Sbjct  37
RGLGNKGTLPWKCNSVDMKYFRSVTTYVDESKYEKLKWKRERYLRMEASQGGGDNTSGGD  96

Query  68
PPTEKKRNAVVMGRKTWESVPVKFRPLKGRLNIVLSSKATVEELLAPLPEGQRAAAAQDV  127
               +K +N VVMGR  WES+P +++PL  R+N+VLS   T E++
+ V
Sbjct  97   NNADKLQNVVVMGRSNWESIPKQYKPLPNRINVVLSKTLTKEDV---------
---KEKV  144

Query  128
VVVNGGLAEALRLLARPLYCSSIETAYCVGGAQVYADAMLSPCIEKLQEVYLTRIYATAP  187
             ++     +++  L   L        + +GGAQVY + +    I   +++Y
TRI    P
Sbjct  145  FII-----DSIDDLLLLLKKLKYYKCFIIGGAQVYRECLSRNLI---
KQIYFTRINGAYP  196

Query  188  ACTRFFP  194
             C  FFP
Sbjct  197  -CDVFFP  202
```

Alignment of dihydrofolate reductase-thymidylate synthase [Leishmania donovani] and dihydrofolate reductase-thymidylate synthase [Plasmodium falciparum]

```
Score =  340 bits (871),  Expect = 1e-97, Method: Compositional
matrix adjust.
 Identities = 152/288 (53%), Positives = 204/288 (71%), Gaps =
3/288 (1%)

Query  232
HEERQYLELIDRIMKTGIVKEDRTGVGTISLFGAQMRFSLRDNRLPLLTTKRVFWRGVCE  291
            H E QYL +I  IM  G  + DRTGVG +S FG  M+F L
PLLTTK++F RG+ E
Sbjct  323  HPEYQYLNIIYDIMMNGNKQSDRTGVGVLSKFGYIMKFDLSQ-
YFPLLTTKKLFLRGIIE  381

Query  292
ELLWFLRGETNAQLLADKDIHIWDGNGSREFLDSRGLTENKEMDLGPVYGFQWRHFGADY  351
            ELLWF+RGETN   L +K++ IW+ NG+REFLD+R L   +
DLGP+YGFQWRHFGA+Y
Sbjct  382
ELLWFIRGETNGNTLLNKNVRIWEANGTREFLDNRKLFHREVNDLGPIYGFQWRHFGAEY  441

Query  352
KGFEANYDGEGVDQIRSIVETIKANPNDRRLLFTAWNPCALQKMALPPCHLLAQFYVNTD  411
                NY+ +GVDQ+++I+  IK +P  RR+L  AWN   L +MALPPCH+L
QFYV
Sbjct  442
TNMYDNYENKGVDQLKNIINLIKNDPTSRRILLCAWNVKDLDQMALPPCHILCQFYV--F  499

Query  412
TSELSCMLYQRSCDMGLGVPFNIASYALLTILIAKATGLRPGELVHTLGDAHVYRNHVGA  471
             +LSC++YQRSCD+GLGVPFNIASY++ T +IA+   L+P + +H
LG+AHVY NH+ +
Sbjct  500
DGKLSCIMYQRSCDLGLGVPFNIASYSIFTHMIAQVCNLQPAQFIHVLGNAHVYNNHIDS  559

Query  472  LKSQLERVPHAFPTLVFKEERQFLEDYELTDMEVIDYVPHPPIKMEMA  519
            LK QL R+P+ FPTL    + + +ED+ ++D  + +YV H  I M+MA
Sbjct  560  LKIQLNRIPYPFPTLKLNPDIKNIEDFTISDFTIQNYVHHEKISMDMA  607


 Score = 77.0 bits (188),  Expect = 2e-18, Method: Compositional
matrix adjust.
```

```
 Identities = 59/213 (28%), Positives = 93/213 (44%), Gaps =
48/213 (23%)
Query  38   GIGDGESIPWRVPE-DMAFFKDQTTLLR----------------------
NKKPPTEKK  73
            G+G+   +PW+    DM +F   TT +                        N
P ++K
Sbjct  39
GLGNKGVLPWKCNSLDMKYFCAVTTYVNESKYEKLKYKRCKYLNKETVDNVNDMPNSKKL  98
Query  74
RNAVVMGRKTWESVPVKFRPLKGRLNIVLSSKATVEELLAPLPEGKRAAAAQDVVVVNDG  133
            +N VVMGR  WES+P KF+PL  R+N++LS     E+
+DV ++N
Sbjct  99   QNVVVMGRTNWESIPKKFKPLSNRINVILSRTLKKEDF------------
DEDVYIIN-K  145
Query  134
LAEALRLLARPPYCSSIETAYCVGGAQVYADAMLSPCVEKLQEVYLTRIYTTAPACTRFF  193
            + + + LL +  Y       + +GG+ VY + +    ++K   +Y TRI +T
C  FF
Sbjct  146  VEDLIVLLGKLNY----YKCFIIGGSVVYQEFLEKKLIKK---
IYFTRINSTY-ECDVFF  197
Query  194  PFPPENTTTAWDLASSQGRRKSEADGLEFEICK  226
            P   EN    + + S      S    L+F I K
Sbjct  198  PEINEN---EYQIISVSDVYTSNNTTLDFIIYK  227

 Score = 18.9 bits (37),  Expect = 0.58, Method: Compositional
matrix adjust.
 Identities = 7/19 (37%), Positives = 12/19 (63%), Gaps = 2/19
(11%)

Query  400  CHL--LAQFYVNTDTSELS  416
            CH+  L +FY N D  +++
Sbjct  259  CHMKKLTEFYKNVDKYKIN  277

 Score = 16.9 bits (32),  Expect = 2.1, Method: Compositional
matrix adjust.
 Identities = 6/20 (30%), Positives = 11/20 (55%), Gaps = 0/20
(0%)
Query  82   KTWESVPVKFRPLKGRLNIV  101
            + + S+  K+ P    LNI+
Sbjct  313  QIYNSLKYKYHPEYQYLNII  332
```

Alignment of dihydrofolate reductase-thymidylate synthase [Leishmania donovani] and dihydrofolate reductase-thymidylate synthase [Plasmodium vivax]

```
Score =  332 bits (851),  Expect = 3e-95, Method: Compositional
matrix adjust.
 Identities = 151/290 (52%), Positives = 205/290 (71%), Gaps =
3/290 (1%)

Query  230
RNHEERQYLELIDRIMKTGIVKEDRTGVGTISLFGAQMRFSLRDNRLPLLTTKRVFWRGV  289
            + H E QYL +I  I+  G  + DRTGVG +S FG  M+F+L +
PLLTTK++F RG+
Sbjct  331  KQHPEYQYLGIIYDIIMNGNKQGDRTGVGVMSKFGYMMKFNLSE-
YFPLLTTKKLFLRGI  389

Query  290
CEELLWFLRGETNAQLLADKDIHIWDGNGSREFLDSRGLTENKEMDLGPVYGFQWRHFGA  349
             EELLWF+RGETN   L +K++ IW+ NG+REFLD+R L   +
DLGP+YGFQWRHFGA
Sbjct  390
IEELLWFIRGETNGNTLLNKNVRIWEANGTREFLDNRKLFHREVNDLGPIYGFQWRHFGA  449

Query  350
DYKGFEANYDGEGVDQIRSIVETIKANPNDRRLLFTAWNPCALQKMALPPCHLLAQFYVN  409
            +Y     NY+ +GVDQ+++++  IK  P  RR++  AWN   L +MAL
PCH+L QFYV
Sbjct  450
EYTNMHDNYEDKGVDQLKNVIHLIKNEPTSRRIILCAWNVKDLDQMALLPCHILCQFYV-  508

Query  410
TDTSELSCMLYQRSCDMGLGVPFNIASYALLTILIAKATGLRPGELVHTLGDAHVYRNHV  469
               +LSC++YQRSCD+GLGVPFNIASY++ T +IA+   L+P + +H
LG+AHVY NHV
Sbjct  509  -
FDGKLSCIMYQRSCDLGLGVPFNIASYSIFTHMIAQVCNLQPAQFIHILGNAHVYNNHV  567

Query  470  GALKSQLERVPHAFPTLVFKEERQFLEDYELTDMEVIDYVPHPPIKMEMA
519
             +LK QL R+P+ FPTL    E + +ED+ ++D  + +YV H  I MEMA
Sbjct  568  DSLKVQLNRIPYPFPTLKLNPEVKNIEDFTISDFTIENYVHHDKITMEMA
617
```

```
 Score = 67.4 bits (163),  Expect = 2e-15, Method: Compositional
matrix adjust.
 Identities = 51/185 (28%), Positives = 82/185 (44%), Gaps =
49/185 (26%)

Query  38   GIGDGESIPWRVPE-DMAFFKDQTTLLRNKK----------------------
-----PP  69
            G+G+  ++PW+    DM +F   TT +   K
Sbjct  38
GLGNKGTLPWKCNSVDMKYFSSVTTYVDESKYEKLKWKRERYLRMEASQGGGDNTSGGDN  97

Query  70
TEKKRNAVVMGRKTWESVPVKFRPLKGRLNIVLSSKATVEELLAPLPEGKRAAAAQDVVV  129
             +K +N VVMGR +WES+P +++PL  R+N+VLS   T EE+
++ V
Sbjct  98   ADKLQNVVVMGRSSWESIPKQYKPLPNRINVVLSKTLTKEEV----------
--KEKVF  144

Query  130
VNDGLAEALRLLARPPYCSSIETAYCVGGAQVYADAMLSPCVEKLQEVYLTRIYTTAPAC  189
            + D + + L LL +          + +GGAQVY + +    +   +++Y TRI
P C
Sbjct  145  IIDSIDDLLLLLKK----LKYYKCFIIGGAQVYRECLSRNLI---
KQIYFTRINGAYP-C  196

Query  190  TRFFP  194
               FFP
Sbjct  197  DVFFP  201
```